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Protein Purification Using PDZ Affinity Chromatography

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INTRODUCTION

PDZ (PSD-95, DiscsLarge, ZO1) domains function in nature as protein binding domains within scaffold and membrane-associated proteins (Cho et al., 1992; Hung and Sheng, 2002; Kornau et al., 1995; Kornau et al., 1997). They comprise approximately 90 residues and make specific, high affinity interactions with complementary C-terminal peptide sequences, with other PDZ domains, and/or with phospholipids (Brenman et al., 1996; Doyle et al., 1996; Ivarsson et al., 2011; Songyang et al., 1997). We have previously shown that the specific, strong interactions of PDZ domains with their ligands make them well suited for use in affinity chromatography (Walkup IV and Kennedy, 2014). Here we provide in-depth protocols for the PDZ affinity chromatography procedure, that are applicable for the purification of proteins that contain PDZ domains or PDZ domain-binding ligands, either naturally or introduced by genetic engineering. We detail the preparation of affinity resins comprised of PDZ domains or PDZ domain peptide ligands coupled to solid supports. These affinity matrices can be used to purify neuronal proteins or protein domains containing endogenous PDZ domains or PDZ ligands, eluting the proteins with free PDZ domain peptide ligands. Proteins of Interest (POIs) lacking endogenous PDZ domains or ligands can be engineered as fusion products containing C-terminal PDZ domain ligand peptides or internal, N or C-terminal PDZ domains, and the fusion proteins can be purified by the same method. Enzymes and fluorescent proteins fused to PDZ domains or PDZ domain ligands purified using this method have been recovered in active form (Walkup IV and Kennedy, 2014).

The Strategic Planning section provides guidelines for selection of PDZ domain and ligand pairs for the PDZ affinity chromatography procedure and includes a list of successfully implemented pairs in Table 1. Design parameters for PDZ domains or ligands for affinity resins, affinity tags or elution agents are also discussed. Basic Protocol 1 describes the preparation of PDZ ligand affinity resin from commercially available N-hydroxysuccinimide (NHS)-Activated Agarose and PDZ domain peptide ligand. Basic Protocol 2 describes the preparation of PDZ domain affinity resin, which requires expression of a PDZ domain, its

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purification utilizing the PDZ ligand affinity resin prepared in Basic Protocol 1, and coupling of the purified domain to NHS-Activated Agarose. Alternate Protocol 2 describes the purification of a PDZ domain and preparation of affinity resin in a single step, utilizing Promega's HaloTag technology (Los et al., 2008; Los and Wood, 2007). Basic Protocol 3 outlines the PDZ affinity chromatography procedure for purification of POIs fused to PDZ affinity tags employing PDZ domain or PDZ ligand affinity resin. Parameters deemed essential to the success of a PDZ affinity chromatography experiment are discussed in the Commentary. Figures 1 and 2 provide an overview of the PDZ affinity chromatography procedure employing PDZ domain affinity resin and PDZ domain peptide ligand affinity resin, respectively.

STRATEGIC PLANNING

Basic Protocol 1

Design of PDZ ligands for coupling to NHS-Activated Agarose—PDZ domain peptide ligands coupled to NHS-Activated Agarose should be six or more amino acids in length. To ensure adequate spacing between the C-terminal ligand sequence and the Agarose beads we incorporate a short linker sequence, GAG, on the amino terminus of each peptide ligand. It is important to select a ligand sequence that binds the specific PDZ domain contained in or fused to the POI. We performed literature searches to identify natural and synthetic ligand sequences capable of binding to the PDZ domain of interest. Table 1 lists PDZ domains and their respective ligand sequences that have been successfully used for PDZ affinity chromatography.

PDZ domain peptide ligands may be synthesized by commercial vendors or core facilities and should be purified to greater than 75% homogeneity before use. We have used commercially synthesized ligands because of their low cost and rapid availability. We have not seen any significant difference in performance of PDZ ligand affinity resins, or in ligand density, among peptides ranging from 75% to greater than 99% purity.

PDZ domains have also been shown to bind phospholipids (Ivarsson et al., 2011), cyclic peptides (Gee et al., 1998; Seedorff et al., 2010), and small molecules (Grandy et al., 2009). While we have not yet prepared affinity resins using these types of molecules, we believe that they could be successfully coupled to resin and used to purify POIs containing PDZ domains.

We provide methods for preparing PDZ ligand affinity resin by coupling the GAGSSIESDV peptide to NHS-Activated Agarose in Basic Protocol 1. This ligand affinity resin can then be used to purify the PDZ2 domain of PSD-95, which can then be coupled to NHS-Activated Agarose to prepare PDZ domain affinity resin (Basic Protocol 2). These two sets of resin can be used to purify POIs fused to PDZ domain affinity tags (PDZ2 domain of PSD-95) or to C-terminal PDZ ligand affinity tags (SSIESDV), respectively (Basic Protocol 3).

Basic Protocol 2

Design of PDZ domains for coupling to NHS-Activated Agarose—PDZ domain cDNA can be codon optimized for expression in *E.coli* (or other hosts) prior to cloning into

a suitable expression vector (Welch et al., 2009a; Welch et al., 2009b; Welch et al., 2011). Codon optimization has increased our PDZ domain expression levels 3 to 10-fold relative to endogenous cDNA sequences (e.g. 150-500 mg vs. 50 mg of protein per liter of *E. coli* cells) and has simplified cloning procedures utilizing restriction enzymes. We have expressed PDZ domains and ligand sequences from T5 or T7 promoters in intellectual property-free pJExpress vectors from DNA2.0.

The cDNAs encoding PDZ domains should be designed to include the entire PDZ domain fold and the 5-10 amino acids N-terminal to the domain. Inclusion of the additional N-terminal amino acids ensures adequate spacing between the N-terminus and the resin during covalent coupling and allows the ligand binding pocket to be accessible to POIs. Ideally, when PDZ domains are coupled to resin with NHS, the N-terminal primary amine will couple to the resin, producing uniformly oriented domains. In reality, primary amines on lysine residues can also couple to the resin, producing heterogeneously oriented PDZ domains. Inclusion of a short N-terminal spacer sequence has improved our coupling efficiency and purification yields, however we haven't directly measured its effect on the heterogeneity of immobilized PDZ domains.

We have successfully used the PDZ domain sequences listed in Table 1 for the preparation of PDZ affinity resin and as PDZ domain affinity tags. If an investigator is interested in employing a PDZ domain not listed in Table 1, we suggest either identifying domain boundaries from the literature or from Uniprot protein sequence database (Ladunga, 2009), or by aligning the sequence in question to a PDZ domain from Table 1 (Notredame, 2010; Simossis et al., 2003). Alternatively, domain boundaries can be selected by constructing a homology model of the PDZ domain using the structure of a PDZ domain listed in Table 1 as a template (Eswar et al., 2006; Webb and Sali, 2014).

We provide a method for expressing the PDZ2 domain of PSD-95, which can be purified using the PDZ ligand affinity resin (GAGSSIESDV) prepared in Basic Protocol 1. The purified PDZ2 domain can be coupled to NHS-Activated Agarose to prepare PDZ domain affinity resin (Basic Protocol 2). This resin can then be used to purify POIs fused to C-terminal PDZ ligand affinity tags (SSIESDV) (Basic Protocol 3).

Design of PDZ ligands for elution of PDZ domains from PDZ ligand affinity resin—In Basic Protocol 2, a PDZ ligand affinity resin is used to purify a recombinantly expressed PDZ domain from a cellular lysate. In Basic Protocol 3, a PDZ domain affinity resin is used to purify a recombinantly expressed POI, fused to a PDZ affinity tag, from a cellular lysate. In both protocols, the PDZ domains or the POIs bound to PDZ affinity resin are eluted with concentrated (200-400 μg/ml) PDZ domain peptide ligand. For this purpose, we have used six amino acid long, natural or synthetic ligand sequences of greater than 75% purity. Peptide ligands to be used for elution should have a dissociation constant (K_D) of 5 μM or less for binding to the PDZ domain affinity resin or PDZ domain affinity tag. It is advisable to performa literature search to identify the most desirable ligand sequences for binding to the PDZ domain of interest. Peptide ligands that we have used successfully for elution of POIs from PDZ affinity resin are listed in Table 1.

The protocols provided here present methods for use of SIESDV or SIETEV peptides for elution of free PDZ2 domains of PSD-95 or PDZ domain affinity tagged POIs (PDZ2 tags) from PDZ ligand affinity resin containing bound GAGSSIESDV peptide. They also provide methods for using these peptides to elute POIs tagged with the C-terminal PDZ ligand affinity tag SSIESDV from PDZ domain affinity resin containing PDZ2 of PSD-95.

Alternate Protocol 2

HaloTag technology is convenient way to covalently couple PDZ domains to resin and results in a uniform orientation of the PDZ domains, which are all linked via an engineered HaloTag to chloroalkane resin. HaloTag-PDZ domain fusion proteins are generated by inserting PDZ domain cDNA (designed as described above for Basic Protocol 2) into the commercially available plasmid pFN18A, in frame with an N-terminal HaloTag protein. This vector encodes a HaloTag-PDZ domain fusion protein that contains a TEV protease site between the HaloTag and the PDZ domain. It is convenient to remove common restriction sites from the coding region during the process of codon optimization for expression in *E. coli*. We used KpnI and PvuI restriction sites in the pFN18A vector and appropriate PCR primers to amplify and clone the cDNAs encoding PDZ domains into the plasmid in frame with the HaloTag cDNA. To clone PDZ domains from endogenous cDNAs that contain KpnI and PvuI restriction sites in unfavorable locations, we suggest the polymerase incomplete primer extension (Klock et al., 2008; Klock and Lesley, 2009) or Gibson assembly (Gibson, 2011; Gibson et al., 2009) cloning methods that circumvent the need for restriction enzymes.

Basic Protocol 3

Expression of POIs with endogenous PDZ domains or ligands—Table 2 lists neuronal proteins containing endogenous PDZ domains or ligands that have been purified by PDZ affinity chromatography. Each POI requires establishment of optimal expression conditions and protein boundaries. We have used several strategies to accomplish soluble expression of POI's that were difficult to express. These have included systematic N and/or C-terminal truncation of protein boundaries (Klock et al., 2008), low temperature expression (Schein, 1989; Vera et al., 2007), co-expression with protein binding partners (Sorensen and Mortensen, 2005) or molecular chaperones (Baneyx and Mujacic, 2004; Ikura et al., 2002; Nishihara et al., 2000), expression in solubility enhancing bacterial strains (Miroux and Walker, 1996), fusion to solubility enhancing tags (Makrides, 1996; Sorensen and Mortensen, 2005), decreased inducer concentration, and alternate promoter systems (Jana and Deb, 2005; Qing et al., 2004).

To date, PDZ affinity chromatography has only been applied to proteins expressed in *E. coli*, which is convenient *because of* the absence of host cell proteins that interact with PDZ affinity matrices (Walkup IV and Kennedy, 2014). We are currently in the process of evaluating the use of these methods for purification of proteins from cell lines that naturally express PDZ domains (e.g. HEK293, Hi-5, CHO cells), and will update this unit when data become available.

Design and Expression of POIs with PDZ affinity tags—As discussed above, each recombinant POI requires establishment of optimal expression conditions and protein boundaries. Our default conditions for expression of a POI fused to a PDZ affinity tag makes use of auto-induction media and expression at 37 °C, unless literature precedent suggests otherwise (Walkup IV and Kennedy, 2014).

PDZ ligand affinity tags can only bind to PDZ domains when placed at the C-terminus of a POI. We have found it useful to insert a short 3 to 5 amino acid linker sequence (e.g. AAA) immediately after the POI, followed by a TEV protease (ENLYFQG) or PreScission protease (LEVLFQGP) cleavage site, and the C-terminal PDZ ligand affinity tag (SSIESDV).

PDZ domain affinity tags can bind either to other PDZ domains or to PDZ ligands when placed at either terminus of a POI, or when placed internally between two proteins (e.g. a POI and a solubility enhancing tag). When PDZ domains are used as N-terminal affinity tags, we include a TEV protease or PreScission protease cleavage sequence and a three to five amino acid linker sequence after the PDZ domain tag, followed by the POI. For C-terminal PDZ domain tags we insert the linker and protease sequences immediately following the POI, to separate the N-terminal POI from the C-terminal PDZ domain affinity tag. The decision where to place internal PDZ domain affinity tags is dependent upon the goals of the investigator. For example, if the investigator wishes to express a POI with both a solubility enhancing tag and a PDZ domain affinity tag, with the goal of purifying an untagged POI, we suggest placing the solubility-enhancing tag upstream of the PDZ domain tag, and including a short linker region and protease site between the PDZ domain tag and the POI.

For trial purposes, we suggest making three tagged variants of each POI: 1) N-terminal PDZ domain affinity tag (e.g. PDZ2 domain of PSD-95), 2) C-terminal PDZ domain affinity tag (PDZ2 domain of PSD-95), and 3) C-terminal PDZ ligand affinity tag (SSIESDV). These three fusion proteins will allow purification of the POI on both PDZ domain (PDZ2 domain of PSD-95) and PDZ ligand affinity resin (GAGSSIESDV). Construction of all three will increase the chance of identifying a successful affinity tag / affinity resin pair for each unique POI.

BASIC PROTOCOL 1

PREPARATION OF PDZ LIGAND AFFINITY RESIN—This protocol describes the synthesis of 10 ml of PDZ ligand affinity resin and is outlined in Figure 3. Preparation of PDZ ligand affinity resin includes the following steps: (1) peptide ligand design and synthesis, (2) coupling of ligand to commercial NHS-Activated Agarose resin*, (3) blocking of unreacted NHS sites, (4) washing and storage of resin, and (5) estimation of the density of the resin ligand. Strategies for design and synthesis of PDZ domain peptide ligands are described in Strategic Planning under Basic Protocol 1. The GAGSSIESDV ligand used in this protocol contains a GAG spacer sequence and a SSIESDV sequence that binds to the PDZ1 and PDZ2 domains of PSD-95. The ligand is added to the NHS-Activated Agarose and the coupling reaction is carried out for 2 or more hours. The remaining reactive groups on the resin are then blocked with ethanolamine. Released NHS and ethanolamine are

washed away with storage buffer, and the resin can be stored at 4 °C for up to a year. The ligand density of the resin is calculated by quantifying with a colorimetric protein assay, the concentrations of the peptide in the reaction supernatants before and after coupling.

*Due to the time consuming, laborious and potentially hazardous nature of preparing chemically activated chromatography media, this protocol recommends commercially available NHS-Activated Agarose. If affinity resin with differing physical or chemical properties is desired, alternative chemically activated supports are available (e.g. Bio-Rad Affi-Gel 10). For preparation of chemically activated chromatography media, we suggest the protocols outlined in (Luong and Scouten, 2008) and (Hermanson, 2013).

Materials

Solutions and Reagents: NHS-Activated Agarose

Ultrapure water

Coupling/Wash Buffer I (see recipe)

300 mg PDZ ligand peptide (75% purity or above) (e.g. GAGSSIESDV)

Peptide Buffer (see recipe)

Quenching Buffer (see recipe)

Storage Buffer I (see recipe)

Special Equipment: End-over-end or rotating wheel mixer

50 ml screw-cap polypropylene tubes

Centrifuge

Couple PDZ ligand to NHS-Activated Agarose

1. Pipette 20 ml of a 50% slurry of NHS-Activated Agarose resin in acetone storage solution into a 50 ml Falcon Tube.
2. Remove the storage solution by centrifugation at 1,000 × g for 1 min at room temperature. Discard the supernatant.
3. Wash the 10 ml of settled NHS-Activated Agarose resin with 30 ml (3 CV) of ultrapure water. Mix gently by inverting the tube until the resin is suspended and no clumps remain.
4. Remove the ultrapure water by centrifugation at 1,000 × g for 1 min at room temperature. Discard the supernatant.
5. Repeat steps 3 and 4, two additional times.
6. Wash the resin with 30 ml (3 CV) of Coupling/Wash Buffer I. Mix gently by inverting the tube until the resin is suspended and no clumps remain.

7. Remove Coupling/Wash Buffer I by centrifugation at 1,000 × g for 1 min at room temperature. Discard the supernatant.

8. Repeat steps 6 and 7, one additional time.

9. Prepare Peptide Coupling Buffer by resuspending peptide to a concentration of 10 mg/ml in Coupling/Wash Buffer I.

   Remove a small aliquot of Peptide Buffer for calculation of ligand density of the resin and coupling efficiency (below).

10. Add 30 ml (3 CV) of Peptide Coupling Buffer to the resin, mix gently until no clumps remain and incubate for 2 hrs at room temperature with continuous agitation on an end-over-end mixer at 30-40 RPM.

    Approximately 80% of the coupling reaction occurs in the first 30 minutes. To further increase efficiency, we extend the reaction to 2 hours. Alternatively, the coupling reaction may be performed overnight at 4 °C.

11. Separate the unbound peptide from the resin by centrifugation at 1,000 × g for 1 min at room temperature. Remove an aliquot of supernatant for calculation of ligand density of the resin and coupling efficiency (below), then discard the remaining supernatant.

Wash, quench and store PDZ ligand affinity resin

12 Wash the resin with 30 ml (3 CV) of Coupling/Wash Buffer I. Mix gently by inverting the tube until the resin is suspended and no clumps remain.

13 Remove Coupling/Wash Buffer I by centrifugation at 1,000 × g for 1 min at room temperature. We have found that the amount of peptide recovered in the wash fractions is less than 5% of the total. However, if desired, remove an aliquot of supernatant for inclusion in the calculation of ligand density and coupling efficiency, then discard the remaining supernatant.

14 Repeat steps 12 and 13, one additional time.

15 Add 30 ml (3 CV) of Quenching Buffer to the resin and incubate for 20 minutes at room temperature with continuous agitation on an end-over-end mixer at 30-40 RPM.

16 Remove Quenching Buffer by centrifugation at 1,000 × g for 1 min at room temperature. Discard the supernatant.

17 Wash the resin with 40 ml (4 CV) of Coupling/Wash Buffer I. Mix gently by inverting the tube until the resin is suspended and no clumps remain.

18 Remove Coupling/Wash Buffer I by centrifugation at 1,000 × g for 1 min at room temperature. Discard the supernatant.

19 Wash the resin with 40 ml (4 CV) of Storage Buffer I. Mix gently by inverting the tube until the resin is suspended and no clumps remain.

20 Remove Storage Buffer I by centrifugation at 1,000 × g for 1 min at room temperature. Discard the supernatant.

21 Repeat steps 19 and 20, one additional time.

22 Resuspend resin in 10 ml (1 CV) of Storage Buffer I and transfer to sterile 50 ml conical tube. Store resin at 4 °C for up to a year.

Determine ligand density of PDZ ligand affinity resin

23 Determine the concentration of the peptide ligand in the starting solution, and in the resin supernatant after coupling, by BCA, 660 nm assay, or absorbance measurements at 280 nm. Calculate the difference between the amount of peptide in the starting buffer and that in the supernatant after coupling and divide by the ml of settled resin (10 ml) to determine the density of ligand on the resin. We express the density as nmol per µl resin (mM).

> The NHS leaving group interferes with the BCA assay and absorbance measurements at 280 nm. Therefore, to measure the concentration of unbound peptide, dialyze samples against PBS before performing BCA assay or absorbance measurements at 280 nm. Alternatively, if the PDZ ligand contains basic residues, peptide concentration can be measured by the Pierce 660 nm protein assay.

24 We have found that ligand densities of PDZ ligand resins vary from 20 to 26 nmol peptide/µl resin (20–26 mM), which represent coupling efficiencies of 74–96%, respectively.

BASIC PROTOCOL 2

PREPARATION OF PDZ DOMAIN AFFINITY RESIN—This protocol describes the synthesis of 5 ml of PDZ domain affinity resin and is outlined in Figure 4. Preparation of PDZ domain affinity resin includes the following steps: (1) design and synthesis of cDNA encoding the PDZ domain, (2) expression of PDZ domain, (3) purification of PDZ domain using PDZ ligand affinity resin, (4) coupling of PDZ domain to commercial NHS-Activated Agarose resin, (5) blocking of unreacted NHS sites, (6) washing and storage of resin, and (7) estimation of ligand density of the resin. Strategy for design and synthesis of cDNA encoding a PDZ domain is discussed in Strategic Planning under Basic Protocol 2. After the cDNA is constructed, it is transformed into *E. coli* for expression of the protein and then the expressed PDZ domain is purified with PDZ ligand affinity resin. The PDZ domain used in this protocol is PDZ2 of PSD-95, which binds to the sequence SSIESDV on the PDZ ligand affinity resin prepared in Basic Protocol 1. The purified PDZ domain is added to the NHS-Activated Agarose and the coupling reaction is carried out for 12-16 hours. The remaining NHS reactive groups on the resin are then blocked with ethanolamine. Excess NHS and ethanolamine are washed away with storage buffer, and the resin is stored at 4 °C for up to six months. The ligand density of the resin is calculated as described above for PDZ-ligand resin, except that SDS-PAGE can be used to quantify the amounts of PDZ domain protein.

Alternate Protocol 2 describes the use of commercially available HaloTag technology to couple the PDZ domain to resin in a single step, circumventing the need to purify PDZ domains prior to coupling. The disadvantage of HaloTag technology is that we have found the resulting affinity resins degrade with a half life of approximately two weeks.

Materials

Solutions and Reagents: BL21(DE3) cells

Expression plasmid containing PDZ domain cDNA

LB Miller Agar

Carbenicillin

LB Miller Broth

1 M IPTG (isopropyl β-D-1-thiogalactopyranoside)

BugBuster Lysis Buffer (see recipe)

Prepared PDZ ligand affinity resin (see Basic Protocol 1)

Purification Buffer (see recipe)

Peptide Elution Buffer (see recipe)

Denaturing Buffer (see recipe)

Storage Buffer II (see recipe)

Coupling/Wash Buffer II (see recipe)

NHS-Activated Agarose

Ultrapure water

Quenching Buffer (see recipe)

Special Equipment: 2 liter Plastic Baffled Erlenmeyer Flasks

Orbital shaker

Centrifuge

End-over-end or rotating wheel mixer

Microfluidizer (Optional)

Plastic or Glass Chromatography Columns

Protein concentrators, 9 kDa Molecular Weight Cutoff

Express PDZ domain

1. Transform BL21(DE3) cells with a plasmid containing cDNA encoding the PDZ domain. Select transformed cells on LB Agar supplemented with 100 μg/ml carbenicillin (or appropriate antibiotic).
2. Select a single colony of BL21(DE3) cells harboring the plasmid and inoculate 5 ml of LB broth supplemented with 100 μg/ml carbenicillin in a 14 ml BD Falcon tube.
3. Grow starter culture overnight (10-12 hours) at 37 °C and 200-225 RPM.
4. Inoculate 500 ml of LB broth containing 100 μg/ml carbenicillin in a 2 liter baffled Erlenmeyer flask with 1 ml of overnight starter culture.
5. Grow culture for 4-5 h at 37 °C and 200-225 RPM, until OD_{600} reaches 1.0.
6. Induce protein expression by adding 1 M IPTG to a final concentration of 0.5 mM.
7. Grow for 4 hours at 37 °C and 200-225 RPM.
8. Harvest cells by centrifugation at 10,000 × g for 10 min at 4 °C, and store cell pellets at -80 °C.

Purify PDZ domain using PDZ ligand affinity resin

9 Resuspend bacterial cell pellets (~20 g) containing fusion proteins in 5 ml of BugBuster Lysis Buffer per gram of cells.

10 Homogenize cell suspension in a Teflon-glass or Dounce homogenizer or triturate with a pipette until no clumps remain. Incubate for 20 minutes at room temperature with continuous agitation on an end-over-end mixer at 30-40 RPM to lyse cells.

> Alternatively, lysis can be performed by microfluidization. Resuspend cell pellets in 10 ml of Purification Buffer supplemented with 25 U/ml Benzonase and 1 kU/ml ReadyLyse, per gram of cells by triturating several times with a pipette or by Teflon-glass or Dounce homogenization until no clumps remain. Lyse cells by three passages through a microfluidizer.

11 Clarify cell lysate by centrifugation at 30,000 × g for 60 min at 4 °C.

12 During the centrifugation, equilibrate PDZ ligand-NHS-Agarose resin with Purification Buffer.

> Split 30 ml of PDZ ligand-NHS-Agarose resin suspension (50% slurry) between five 50 ml polypropylene tubes. Spin at 1,000 × g for 5 minutes at room temperature. Discard supernatant. Equilibrate resin by adding 45 ml of Purification Buffer per tube and mix gently by inverting the tube until the resin is suspended and no clumps remain. Spin at 1,000 × g for 5 minutes at room temperature. Discard supernatant. Repeat equilibration and centrifugation step once more.

If using microfluidization for lysis, split slurry between ten 50 ml polypropylene tubes and equilibrate as described in the previous paragraph.

13 Add clarified lysate to equilibrated PDZ ligand-NHS-Agarose resin and mix with continuous agitation for 1 hour at 4 °C on an end-over-end mixer.

We observe maximal binding of PDZ domains to PDZ ligand-NHS-Agarose when incubating for 60-90 minutes. Extending the incubation time to 2 to 4 hours resulted in decreased yield of bound PDZ domains.

14 Separate unbound PDZ domains from resin by centrifugation at 1,000 × g for 5 min at 4 °C.

15 Resuspend the resin in 15 ml (1 CV) of Purification Buffer and transfer to a, chromatography column with the outlet closed by a stopper. Allow the resin to settle for 60 min before removing the stopper to drain the column.

16 Wash the resin with 300 ml (20 CV) of Purification Buffer.

17 Elute PDZ domains by application of 60 ml (4 CV) of Peptide Elution Buffer.

18 Pipette eluted solution containing PDZ domains into a 20 ml 9 kDa MWCO protein concentrator and centrifuge in a swinging bucket rotor at 3,000 × g for 25 min at 4 °C.

It is imperative that you use a swinging bucket rotor. We have found that attempts to concentrate using a fixed angle rotor resulted in leakage from the concentrator and loss of sample.

19 Repeat step 18 as needed until the final volume of the eluate is reduced to 10 ml. The resulting protein concentration should be approximately 10-20 mg/ml (500-1400 μM).

While concentrating the eluted PDZ domain, wash and regenerate the PDZ ligand affinity resin. Apply 120 ml (4 CV) of Denaturing Buffer to PDZ ligand affinity resin at room temperature. Then renature the immobilized PDZ ligand by washing resin with 450 ml (15 CV) of Storage Buffer II at room temperature. Resuspend resin in 30 ml (1 CV) of Storage Buffer II, and store at 4 °C. We observed no loss of binding capacity after 5 cycles of elution, denaturation and renaturation or after storage for 1 year at 4 °C.

20 Dilute concentrated eluate to 20 ml with Coupling/Wash Buffer II in preparation for coupling to NHS-Activated Agarose, and centrifuge again in the concentrator in a swinging bucket rotor at 3,000 × g for 25 min at 4 °C.

21 Repeat step 20 at least five times to exchange the PDZ domains into Coupling/Wash Buffer II, ending with 10 ml of retentate.

Steps 20 and 21 also serve to remove bound peptide from the PDZ domain. Dialysis is a suitable alternative for buffer exchange and

peptide removal. Dialyze the concentrated retentate from step 19 against 1000 ml of Coupling/Wash Buffer II at 4 °C using dialysis tubing with a 5 kDa or lower MWCO. Exchange the buffer every four to six hours, performing a maximum of four to five buffer changes.

Couple PDZ domain to NHS-Activated Agarose

22 Pipette 10 ml of a 50% slurry of NHS-Activated Agarose resin in acetone storage solution into a 50 ml Falcon Tube.

23 Separate the resin from the storage solution by centrifugation at 1,000 × g for 1 min at room temperature. Discard the supernatant.

24 Wash the 5 ml of settled NHS-Activated Agarose resin with 15 ml (3 CV) of ultrapure water. Mix gently by inverting the tube until the resin is suspended and no clumps remain.

25 Remove the ultrapure water by centrifugation at 1,000 × g for 1 min at room temperature. Discard the supernatant.

26 Repeat steps 24 and 25, two additional times.

27 Wash the resin with 15 ml (3 CV) of Coupling/Wash Buffer II. Mix gently by inverting the tube until the resin is suspended and no clumps remain.

28 Remove Coupling/Wash Buffer II by centrifugation at 1,000 × g for 1 min at room temperature. Discard the supernatant.

29 Repeat steps 27 and 28, one additional time.

30 Add 10 ml (2 CV) of purified PDZ Domain to the resin, mix gently until no clumps remain and incubate for 12-16 hours at 4 °C with continuous agitation on an end-over-end mixer at 30-40 RPM.

Remove a small aliquot of the Purified PDZ domain solution prior to coupling for calculation of ligand density and coupling efficiency.

Approximately 80% of the coupling reaction occurs in the first 30 minutes. To further increase efficiency, we extend the reaction to 12-16 hours at 4 °C.

31 Separate the unbound PDZ domains from the resin by centrifugation at 1,000 × g for 1 min at room temperature. Remove an aliquot of supernatant for calculation of ligand density and coupling efficiency, then discard the remaining supernatant.

Wash, quench and store PDZ domain affinity resin

32 Wash the resin with 15 ml (3 CV) of Coupling/Wash Buffer II. Mix gently by inverting the tube until the resin is suspended and no clumps remain.

33 Remove Coupling/Wash Buffer II by centrifugation at 1,000 × g for 1 min at room temperature. Remove an aliquot of supernatant for calculation of ligand density and coupling efficiency, then discard the remaining supernatant.

34 Repeat steps 32 and 33, one additional time.

35 Add 15 ml (3 CV) of Quenching Buffer to the resin and incubate for 20 minutes at room temperature with continuous agitation on an end-over-end mixer at 30-40 RPM.

36 Remove Quenching Buffer by centrifugation at 1,000 × g for 1 min at room temperature. Discard the supernatant.

37 Wash the resin with 20 ml (4 CV) of Coupling/Wash Buffer II. Mix gently by inverting the tube until the resin is suspended and no clumps remain.

38 Remove Coupling/Wash Buffer II by centrifugation at 1,000 × g for 1 min at room temperature. Discard the supernatant.

39 Wash the resin with 20 ml (4 CV) of Storage Buffer II. Mix gently by inverting the tube until the resin is suspended and no clumps remain.

40 Remove Storage Buffer II by centrifugation at 1,000 × g for 1 min at room temperature. Discard the supernatant.

41 Repeat steps 39 and 40, one additional time.

42 Resuspend resin in 5 ml (1 CV) of Storage Buffer II and transfer to a sterile 50 ml conical tube. Store resin at 4 °C for up to six months.

Determine ligand density of PDZ domain affinity resin

43 Determine the coupling efficiency of the conjugation reaction and the density of immobilized PDZ domains on the resin by SDS-PAGE. Calculate the difference between the amount of protein in the starting buffer and that remaining in the supernatant after coupling, and divide by the ml of settled resin (10 ml) to determine the density of PDZ domains on the resin. We express the density as pmol per µl resin.

44 Ligand densities of PDZ domain affinity resin vary from 600 to 1300 pmol PDZ domain /µl resin (600 - 1300 µM), which represents coupling efficiencies of 50–90%, respectively.

ALTERNATE PROTOCOL 2

PREPARATION OF PDZ DOMAIN AFFINITY RESIN USING HALOTAG TECHNOLOGY—This protocol describes the synthesis of 10 ml of PDZ domain-HaloTag-HaloLink ligand affinity resin and is outlined in Figure 5. The preparation includes the following steps: (1) design and synthesis of cDNA encoding a fusion of a HaloTag with a PDZ domain, (2) expression of fusion proteins, (3) purification and covalent coupling of HaloTag-PDZ fusion proteins to HaloLink resin, (4) washing and storage of resin, and (5) estimation of ligand density of the resin. The design and synthesis of HaloTag-PDZ domain cDNA is discussed in Strategic Planning under Basic Protocol 2 and Alternative Protocol 2. The cDNA encoding a HaloTag-PDZ domain fusion proteinis transformed into *E. coli* for expression of the protein. After lysis of the bacteria, the HaloTag-PDZ protein is purified and covalently coupled to HaloLink resin in a single step. The PDZ domain-HaloTag-

HaloLink resin is then washed with storage buffer and stored at 4 °C. The ligand density of the resin is calculated by quantifying the concentration of PDZ domain released by TEV proteolysis of an aliquot of the resin by SDS-PAGE with Coomassie staining.

This alternate protocol combines purification of the PDZ domain and covalent linkage to a solid support in a single step, eliminating the the need for pre-purification of the PDZ domain before coupling to NHS-Activated Agarose resin (Basic Protocol 2). Additionally, this procedure produces affinity columns that have PDZ domains in a uniform orientation unlike NHS-coupled affinity columns which contain PDZ domains in heterogenous orientations. The major disadvantages of this method are the high cost of HaloLink resin and the short lifespan of the resulting affinity resin. We have found that the covalent linkage between the PDZ domain-HaloTag fusion protein and HaloLink resin is unstable under our storage conditions, resulting in a decline in ligand density (approximately 75% loss of ligand density in 30 days at 4 °C).

Additional Materials (also see Basic Protocol 2)

Solutions and Reagents: pFN18A plasmid containing HaloTag-PDZ domain fusion protein

Overnight Express Instant Terrific Broth (EMD Millipore)

HaloTag Lysis Buffer (see recipe)

HaloLink Resin (Promega)

HaloTag Purification Buffer (see recipe)

HaloTag Storage Buffer (see recipe)

HaloTag Cleavage Buffer (see recipe)

Express HaloTag - PDZ domain fusion proteins

1. Transform BL21(DE3) cells with a pFN18A plasmid containing cDNA encoding a HaloTag-PDZ domain fusion protein. Select transformed cells on LB Agar supplemented with 100 μg/ml carbenicillin.
2. Select a single colony of BL21(DE3) cells harboring the pFN18A plasmid and inoculate 5 ml of LB broth supplemented with 100 μg/ml carbenicillin.
3. Grow starter culture overnight (10-12 hours) at 37 °C and 200-225 RPM.
4. Inoculate 500 ml of Overnight Express Instant Terrific Broth (TB) Media containing 100 μg/ml carbenicillin with 1 ml of overnight starter culture.
5. Grow culture for 16 h at 37 °C with shaking at 200-225 RPM.
6. Harvest cells by centrifugation at 10,000 × g (4 °C), and store cell pellets at -80 °C.

   We have found it convenient to divide the pellets into aliquots of 5 g or less before freezing, and scale down the preparation accordingly, when we

choose to use HaloTag-HaloLink resin and need to make smaller batches of resin every few weeks.

Couple HaloTag - PDZ domain fusion proteins to HaloLink resin

7 Resuspend bacterial cell pellets (~10 g) containing fusion proteins in 5 ml of HaloTag Lysis Buffer per gram of cells.

8 Homogenize cell suspension in a Teflon-glass or Dounce homogenizer or triturate with a pipette until no clumps remain and then incubate for 20 min at room temperature with continuous agitation on an end-over-end mixer at 30-40 RPM to lyse cells.

9 Clarify cell lysate by centrifugation at 30,000 × g for 60 min at 4 °C.

10 During centrifugation, equilibrate HaloLink resin with HaloTag Purification Buffer.

Split 40 ml of HaloLink resin suspension (25% slurry) between two 50 ml polypropylene tubes. Spin at 2,000 × g for 5 minutes at room temperature. Discard supernatant. Equilibrate HaloLink resin by adding 40 ml of HaloTag Purification Buffer per tube and mix gently by inverting the tube until the resin is suspended and no clumps remain. Centrifuge at 2,000 × g for 5 min at room temperature. Discard supernatant. Repeat equilibration and centrifugation steps once more before performing step 11.

11 Add clarified lysate to equilibrated HaloLink resin and mix with continuous agitation for 1.5 hours at 4 °C on an end-over-end mixer.

The HaloTag forms a covalent linkage with the HaloLink resin; therefore, binding times may be extended beyond 1.5 hours to increase the amount of bound HaloTag-PDZ domain. We have extended binding times to two hours without any noticeable degradation of HaloTag-PDZ domain protein.

12 Remove unbound protein by centrifugation at 2,000 × g for 5 min at 4 °C.

13 Resuspend the PDZ-HaloTag-HaloLink resin in 10 ml (1 CV) of Purification Buffer and transfer to a chromatography column with the outlet closed by a stopper. Allow the resin to settle for 60 min before removing the stopper to drain the column.

14 Wash the resin with 200 ml (20 CV) of Purification Buffer, followed by 200 ml (20 CV) of HaloTag Storage Buffer.

15 Resuspend the resin in 10 ml (1 CV) of HaloTag Storage Buffer, and store at 4 °C for up to a month.

Determine ligand density of PDZ domain - HaloTag - HaloLink resin by TEV proteolysis

16 Transfer 200 µl of PDZ domain-HaloTag-HaloLink resin suspension (50%) to a 1.5 ml microfuge tube.

17 Add 1 ml (10 CV) of HaloTag Cleavage Buffer lacking TEV protease.

18 Mix by gently pipetting, then centrifuge tube at 4,000 × g for 2 min at room temperature.

19 Remove supernatant and repeat steps 17 and 18 one more time.

20 Add 200 µl (2 CV) of HaloTag Cleavage Buffer containing 0.3 Units/ml Promega ProTEV Plus or 0.2 µg/µl Sigma TEV Protease to the resin.

21 Mix by gently pipetting, and then transfer to a 0.6 ml microfuge tube.

22 Cap the tube, and incubate at room temperature to 30 °C for 2 hours with continuous agitation on an end-over-end mixer at 30-40 RPM.

23 Centrifuge the tube at 4,000 × g for 2 min at room temperature.

24 Transfer 200 µl (2 CV) of the supernatant to a 1.5 ml microfuge tube labelled “TEV”.

25 Add 200 µl (2 CV) of HaloTag Cleavage Buffer to the resin, resuspend by gently pipetting.

26 Centrifuge the tube at 4,000 × g for 2 min at room temperature.

27 Transfer 200 µl (2 CV) of supernatant to the 1.5 ml microfuge tube containing the supernatant from Step 24.

28 Fractionate aliquots of the combined supernatants by SDS-PAGE and stain with Coomassie Blue. Bound PDZ domains that were released from the resin by proteolysis will appear as a band at approximately 9 to 11 kDa. The size of TEV protease bands will vary depending on the manufacturer. For Promega ProTEV Plus, monomeric and dimeric TEV will appear as bands at approximately 50 and 100 kDa, respectively. Determine the amount of released PDZ domain by measuring the optical density of the band with a scanner and comparing them to standards of known amounts of BSA or lysozyme.

This coupling procedure produces PDZ domain affinity resin with a ligand density of 100 - 200 pmol PDZ domain /µl of resin (100 - 200 µM).

BASIC PROTOCOL 3

PURIFICATION OF POIs USING PDZ AFFINITY CHROMATOGRAPHY—This protocol describes the purification of a POI by a combination of gravity-flow and batch chromatography with 1 ml of PDZ domain or ligand affinity resin (referred to as PDZ affinity resin). PDZ affinity chromatography is performed by incubating a partially purified POI containing a PDZ domain or ligand (naturally occurring or introduced by genetic

engineering), with PDZ affinity resin (Figure 1 and 2). The resin containing bound protein is then transferred to a gravity flow column, and nonspecifically bound proteins are washed from the resin while the POI is retained. The POI is eluted by incubation with wash buffer containing free PDZ ligand. The eluted POI is dialyzed or its buffer is exchanged on a concentrator to remove the PDZ ligand. Purified protein is flash frozen in liquid nitrogen for storage. This procedure can be scaled linearly when larger amounts of purified POI are required.

Materials

Solutions and Reagents: PDZ affinity resin (see Basic Protocol 1 or 2 or Alternate Protocol 2)

Purification Buffer (see recipe) or other column buffer

Partially purified POI or clarified lysate containing POI in Purification Buffer or other column buffer

Peptide Elution Buffer (see recipe)

Denaturing Buffer (see recipe)

Storage Buffer II (see recipe)

Special Equipment: Disposable chromatography column

Centrifuge

Liquid nitrogen

Purify POI on PDZ affinity resin

1. Pipette 2 ml of a 50% slurry of PDZ affinity resin in Storage Buffer I or II into a 15 or 50 ml Falcon Tube (see Step 6).
2. Remove the storage solution by centrifugation at 1,000 × g for 1 min at room temperature. Discard the supernatant.
3. Wash the settled PDZ affinity resin with 14 ml (14 CV) of Purification Buffer. Mix gently by inverting the tube until the resin is suspended and no clumps remain.
4. Remove the Purification Buffer by centrifugation at 1,000 × g for 1 min at room temperature. Discard the supernatant.
5. Repeat steps 4 and 5, one additional time.
6. Add partially purified POI or clarified lysate containing the POI (14 - 50 ml) to 1 ml of equilibrated PDZ affinity resin and mix with continuous agitation for 1 hour at 4 °C on an end-over-end mixer.

   The choice of buffer conditions and lysis method for the preparation of lysate containing POI or partial purification of POI is dependent upon the

POI. We have successfully purified POIs with PDZ affinity chromatography using several different buffers (unpublished observation), but have the most experience with HEPES.

We observe maximal binding of POIs to PDZ affinity resin when incubating for 60-90 min. Extending incubation time to 2-4 hours resulted in decreased yield of bound POIs.

7. Separate unbound protein from the resin by centrifugation at 1,000 × g for 2 min at 4 °C.
8. Remove the supernatant.
9. Resuspend the resin in 2 ml (2 CV) of Purification Buffer and transfer to a chromatography column with the outlet closed by a stopper. Allow the resin to settle for 15 min before removing the stopper to drain the column.
10. Wash the resin with 20 ml (20 CV) of Purification Buffer.
11. Elute POI by application of 4 ml (4 CV) of Peptide Elution Buffer.

    The concentration of the eluted POI can be increased by decreasing the volume of buffer added for elution, increasing the concentration of peptide from 400 to 800 μg/ml, and/or by incubating the column resin with Peptide Elution Buffer without flow for 20 min before collecting the eluate.

12. Pipette eluate into a 9 kDa MWCO protein concentrator and centrifuge in a swinging bucket rotor at 3,000 × g for 25 min at 4 °C. Discard filtrate.
13. Dilute retentate with Purification Buffer, and centrifuge in a swinging bucket rotor at 3,000 × g for 25 min at 4 °C. Discard filtrate.
14. Repeat step 8 at least five times to remove residual bound peptide remaining from the elution.

    Steps 14 and 15 serve to remove bound peptide from the PDZ domain. A suitable alternative for buffer exchange and peptide removal is dialysis. Dialyze the concentrated retentate from step 19 against 1000 ml of Purification Buffer at 4 °C using dialysis tubing with a 5 kDa or lower MWCO. Exchange the buffer every four to six hours, performing a maximum of four to five buffer changes.

    When purifying POIs fused to a PDZ domain or ligand affinity tag, we have opted to leave the affinity tags attached to the POI, as we have not detected any deleterious effects of the tags on POI activity. If removal of affinity tags is necessary, we suggest TEV or PreScission protease.

15. Quick-freeze aliquots of purified POI in liquid nitrogen and store at -80 °C.

Regenerate PDZ affinity resin

16 Remove the remaining bound protein (usually less than 10% of bound protein before elution) and protein contaminants from the resin by applying 4 ml (4 CV) of Denaturing Buffer at room temperature.

17 Renature immobilized PDZ domains or ligands by washing the resin with 15 ml (15 CV) of Storage Buffer II at room temperature.

18 Resuspend the resin in 1 ml (1 CV) of Storage Buffer II, and store at 4 °C.

We observed no loss of binding capacity after 5 cycles of elution, denaturation and renaturation or after storage for 6 months (PDZ domain affinity resin) or 1 year (PDZ ligand affinity resin) at 4 °C. However, the PDZ domain-HaloTag-HaloLink gradually reversed with significant loss of ligand by approximately 30 days.

REAGENTS AND SOLUTIONS—1 M NaH_2PO_4, pH 7.2

5 M NaCl

2% NaN_3 (w/v)

1 M HEPES, pH 7.5

1 M TCEP

0.5 M EDTA

1 M $MgCl_2$

100 mM phenylmethylsulfonyl fluoride (PMSF)

1 M IPTG

BugBuster Lysis Buffer: 1x BugBuster (Prepare using unbuffered 10x BugBuster reagent)

50 mM HEPES, pH 7.5

150 mM NaCl

5 mM TCEP

0.5 mM EDTA

10 mM $MgCl_2$

25 U/ml Benzonase (EMD Millipore)

1 kU/ml ReadyLyse (Epicentre)

2 mM PMSF

Complete Protease Inhibitor (Roche)

Prepare buffer with HEPES, NaCl, TCEP, EDTA and $MgCl_2$ up to 24 hours before use. pH and store at 4 °C. Immediately before use, add 10x BugBuster, Benzonase, ReadyLyse, PMSF and Complete Protease Inhibitor. Discard any unused buffer.

Coupling/Wash Buffer I: 100 mM NaH_2PO_4, pH 7.2

150 mM NaCl

Prepare buffer up to 24 hours before use; pH at room temperature. Store at room temperature for up to a week.

Coupling/Wash Buffer II: 50 mM HEPES, pH 7.5

150 mM NaCl

Prepare buffer up to 24 hours before use; pH at 4 °C. Store at 4 °C for up to a month.

Denaturing Buffer: 50 mM HEPES, pH 7.5

8 M Urea

Prepare buffer up to 24 hours before use. pH and store at 4 °C. Discard any unused buffer.

HaloTag Cleavage Buffer: 50 mM HEPES, pH 7.0

150 mM NaCl

5 mM TCEP

0.5 mM EDTA

2 mM ATP

Promega ProTEV Plus (0.3 units/μl) or Sigma TEV Protease (0.2 μg/μl)

Prepare buffer with HEPES, NaCl, TCEP and EDTA up to 24 hours before use. pH and store at 4 °C. Immediately before use add ATP and TEV protease. Discard any unused buffer.

HaloTag Lysis Buffer: 1x BugBuster (Prepare using unbuffered 10x BugBuster reagent)

50 mM HEPES, pH 7.5

150 mM NaCl

5 mM TCEP

0.5 mM EDTA

10 mM $MgCl_2$

2 mM PMSF

2 mM ATP

25 U/ml Benzonase (EMD Millipore)

1 kU/ml ReadyLyse (Epicentre)

Complete Protease Inhibitor (Roche)

Prepare buffer with HEPES, NaCl, TCEP, EDTA and $MgCl_2$ up to 24 hours before use. pH and store at 4 °C. Immediately before use add 10x BugBuster, ATP, Benzonase, ReadyLyse, PMSF and Complete Protease Inhibitor. Discard any unused buffer.

HaloTag Purification Buffer: 50 mM HEPES, pH 7.5

150 mM NaCl

5 mM TCEP

0.5 mM EDTA

10 mM $MgCl_2$

2 mM PMSF

2 mM ATP

Complete Protease Inhibitor (Roche)

Prepare buffer with HEPES, NaCl, TCEP, EDTA and $MgCl_2$ up to 24 hours before use. pH and store at 4 °C. Immediately before use add ATP, PMSF and Complete Protease Inhibitor. Discard any unused buffer.

HaloTag Storage Buffer: 50 mM HEPES, pH 7.5

150 mM NaCl

5 mM TCEP

0.5 mM EDTA

Complete Protease Inhibitor (Roche)

0.05% NaN_3

Prepare buffer with HEPES, NaCl, TCEP and EDTA up to 24 hours before use. pH and store at 4 °C. Immediately before use add NaN_3 and Complete Protease Inhibitor. Resin in this buffer can be stored up to 3 months before buffer should be replaced with a fresh stock;

however we suggest discarding PDZ domain-HaloTag-HaloLink resin and associated buffer after 1 month because of the decline in ligand density.

Purification Buffer: 50 mM HEPES, pH 7.5

150 mM NaCl

5 mM TCEP

0.5 mM EDTA

10 mM $MgCl_2$

2 mM PMSF

Complete Protease Inhibitor (Roche)

Prepare buffer with HEPES, NaCl, TCEP, EDTA and $MgCl_2$ up to 24 hours before us. pH and store at 4 °C. Immediately before use add PMSF and Complete Protease Inhibitor. Discard any unused buffer.

Peptide Coupling Buffer: 100 mM NaH_2PO_4, pH 7.2

150 mM NaCl

10 mg/ml PDZ domain peptide ligand (e.g. GAGSSIESDV)

Prepare buffer with HEPES and NaCl up to 24 hours before use. pH and store at room temperature. Before use add PDZ domain peptide ligand. Store at room temperature for up to a week.

Peptide Elution Buffer: 50 mM HEPES, pH 7.5

150 mM NaCl

5 mM TCEP

2 mM PMSF

200-400 μg/ml free PDZ domain ligand (e.g. SIESDV)

Complete Protease Inhibitor (Roche)

Prepare buffer with HEPES, NaCl and TCEP up to 24 hours before use. pH and store at 4 °C. Immediately before use add peptide, PMSF and Complete Protease Inhibitor. Unused buffer can be stored for up to 1 month at 4 °C.

Quenching Buffer: 1 M ethanolamine, pH 7.4

Adjust pH to 7.4 with HCl at room temperature. Filter sterilize and store in the dark at room temperature for up to six months.

Storage Buffer I: 100 mM NaH_2PO_4, pH 7.2

150 mM NaCl

0.05% NaN_3

Prepare buffer up to 24 hours before use. pH and store at 4 °C. Resin in this buffer can be stored up to 12 months before buffer should be replaced with a fresh stock.

Storage Buffer II: 50 mM HEPES, pH 7.5

150 mM NaCl

5 mM TCEP

0.5 mM EDTA

10 mM $MgCl_2$

2 mM PMSF

0.05% NaN_3

Complete Protease Inhibitor (Roche)

Prepare buffer with HEPES, NaCl, TCEP, EDTA and $MgCl_2$ up to 24 hours before use. pH and store at 4 °C. Immediately before use add NaN_3, PMSF and Complete Protease Inhibitor. Resin in this buffer can be stored up to 3 months before buffer should be replaced with a fresh stock.

COMMENTARY

Background Information

PDZ domains are ubiquitous, small (~90 residue), compact, modular protein-binding domains that hold together and organize membrane-associated signal transduction complexes (Cho et al., 1992; Hung and Sheng, 2002; Kornau et al., 1995; Kornau et al., 1997). They are often found in multi-domain scaffold proteins that link together large molecular complexes at specific locations within cells. PDZ domains contain a ligand binding pocket that binds short peptide motifs located at the C-termini of proteins (Doyle et al., 1996; Songyang et al., 1997) (Figure 6A-B). Some have also been shown to bind phospholipids and other PDZ domains (Brenman et al., 1996; Ivarsson et al., 2011). They can be divided into three distinct classes based on their specificity for C-terminal peptide sequences (Hung and Sheng, 2002; Songyang et al., 1997). Type I PDZ domains bind C-terminal X-S/T-X-V/I/L peptide motifs, where X denotes any amino acid. Type II PDZ domains bind C-terminal X-U-X-U peptide motifs, where U denotes the bulky, hydrophobic

residues V/Y/F/L/I. Type III PDZ domains bind C-terminal X-D/E-X-V/L peptide motifs. The affinities of PDZ domains for their ligands range from 0.1 to 10 μM (Harris et al., 2001; Lim et al., 2002; Lim et al., 2003; Marfatia et al., 1996; Muller et al., 1996; Niethammer et al., 1998; Songyang et al., 1997; Wang et al., 2005).

In addition to binding C-terminal peptide motifs, certain PDZ domains, such as the neuronal nitric oxide synthase (nNOS) PDZ domain, can bind to other PDZ domains via a β-hairpin motif that immediately follows their core domain (Figure 6C-D). The β-hairpin motif of nNOS binds to the ligand binding pocket of the PDZ domain from syntrophin (Adams et al., 1993) and the PDZ2 domain from PSD-95 to form a complex in which the binding pocket of the nNOS PDZ domain remains unoccupied and capable of binding to an additional PDZ domain ligand (Hillier et al., 1999; Tochio et al., 1999).

Because of the specificity and high affinity of PDZ domains for their C-terminal ligands and for PDZ domains containing β-hairpin ligands, we reasoned that these interacting pairs could provide the core components of an affinity chromatography system including affinity resins, affinity tags, and elution agents. Previously, the PDZ1 domain of the *Drosophila* InaD protein was linked to a support resin and used to purify proteins engineered to contain the NorpA peptide ligand (Kimple and Sondek, 2002). This ligand includes a cysteine residue that attaches covalently via a disulfide bond to the InaD ligand binding pocket. Proteins were eluted from the InaD affinity column by exposure to the reducing reagent DTT.

We have previously shown that affinity columns containing the PDZ domains of PSD-95 can be used to purify active PDZ domain-binding proteins to very high purity in a single step without disulfide bond formation (Walkup IV and Kennedy, 2014). Using solid supports derivatized with recombinant PDZ domains from PSD-95 and elution with synthetic peptides having the sequences of cognate PDZ domain ligands, we purified five heterologously expressed neuronal proteins that contain endogenous PDZ domain ligands. We showed that addition of PDZ domain-related affinity tags to POIs that did not contain endogenous PDZ domains or ligands enabled purification of the POIs on the affinity resins. We used peptides derived from the C-terminal PDZ domain ligand of the N-methyl-D-aspartate type glutamate receptor (NMDAR) subunit GluN2B, PDZ domains from PSD-95, and the nNOS PDZ β-hairpin domain (PDZbh) to construct affinity tags and corresponding affinity resins. We verified that the GluN2B C-terminal PDZ domain ligand tag and N-terminal PSD-95 PDZ2 domain tags did not alter protein activity by fusing PDZ domain-derived affinity tags to DasherGFP, β-Galactosidase (LacZ), and chloramphenicol acetyltransferase (CAT) and assaying their activity using standardized spectrophotometric and fluorescence assays. Unfortunately, nNOS PDZ β-hairpin affinity tagged CAT and LacZ did not bind to PDZ affinity resin, while the nNOS PDZ β-hairpin affinity tag inhibited the proper folding and activity of DasherGFP (Walkup IV and Kennedy, 2014).

The PDZ affinity chromatography system adds an additional set of tools to the toolbox of affinity tags and affinity resins available for protein purification. When compared to the His-Tag system, the C-terminal PDZ ligand affinity tag and PDZ domain affinity resin has a higher affinity for its binding partner (0.1 to 1 μM (Lim et al., 2002; Lim et al., 2003; Wang et al., 2005) vs. 1– 13 μM (Dorn et al., 1998; Guignet et al., 2004; Khan et al., 2006; Lata et

al., 2005; Nieba et al., 1997)), can be eluted from resin with a solution of concentrated peptide without the possibility of metal ion leakage off the column, and does not show detectable background binding to *E. coli* host cell proteins (Bolanos-Garcia and Davies, 2006; Robichon et al., 2011). Like the HisTag, the PDZ ligand affinity tag is relatively small and rarely affects protein function. Unlike the His-Tag system, PDZ affinity chromatography cannot be carried out under denaturing conditions. Additionally, unlike the HisTag that can be used N-terminally, internally or C-terminally, the PDZ ligand affinity tag can only be used C-terminally (PDZ domain affinity tags can be used N-terminally, internally or C-terminally). When compared to the GST Tag system, the PDZ domain affinity tag has a higher affinity for its free ligand (0.1–1 μM (Lim et al., 2002; Lim et al., 2003; Wang et al., 2005) vs. 40–170 μM (Fabrini et al., 2009; Ivanetich and Goold, 1989; Ivanetich et al., 1990; Ivanetich et al., 1988)), can be used in the presence of reducing agents and is monomeric (GST is dimeric or multimeric). Unlike the GST tag, PDZ affinity tags do not seem to have any solubility enhancing effects (Unpublished observation). Additionally, because of their small size, both PDZ domain (10 kDa) and PDZ ligand affinity tags (0.6 kDa), are less likely to affect protein function than the GST tag (26 kDa) (Smith, 2000; Smith and Johnson, 1988), or other large tags including the MBP tag (45 kDa) (di Guan et al., 1988; Maina et al., 1988) and protein A (45 kDa) (Sambrook et al., 1989). For additional information about the properties of affinity tags for protein purification we refer the reader to (Kimple et al., 2013; Malhotra, 2009; Young et al., 2012).

Critical Parameters

Preparation of PDZ ligand affinity resin—PDZ domain peptide ligands coupled to chemically activated resin must be greater than 75% pure, six or more amino acids in length (e.g. SSIESDV) and contain a short linker sequence, e.g. GAG, on their amino terminus. It is imperative to select a ligand sequence that binds the specific PDZ domain fused to the POI.

Due to the time consuming, laborious and potentially hazardous nature of preparing chemically activated chromatography media, commercially available NHS-Activated Agarose should be used for coupling to PDZ ligands. To ensure adequate coupling efficiency, coupling must occur in amine free buffer. Following ligand coupling, remaining NHS esters must be quenched with 1 M ethanolamine before the PDZ ligand affinity resin is washed and stored. During the coupling procedure, small aliquots of supernatant fractions must be collected for determination of ligand density of the resin and coupling efficiency. The NHS group released during coupling interferes with the BCA assay and absorbance measurements at 280 nm. To measure the concentration of unbound peptide present in supernatant fractions, samples must be dialyzed against PBS before performing the BCA assay or absorbance measurements at 280 nm. Alternatively, if the PDZ ligand contains basic residues, peptide concentration can be measured by the Pierce 660 nm protein assay.

Preparation of PDZ domain affinity resin—cDNA encoding a PDZ domain to be used for heterologous expression should be codon optimized for expression in *E.coli* and must be designed to include the entirety of the PDZ domain fold plus 5-10 amino acids N-terminal to the domain. The additional N-terminal amino acids ensure adequate spacing between the N-

terminus of the PDZ domain and the resin during covalent coupling and ensure the PDZ domain ligand binding pocket is accessible to POIs.

Cells containing expressed PDZ domains should be lysed by BugBuster detergent lysis or microfluidization before clarification by centrifugation. To purify the PDZ domain, clarified lysate containing PDZ domains must be incubated with PDZ ligand-Agarose resin for 60-90 min. Extending incubation times to 2 to 4 hours results in decreased yields of bound PDZ domains.

Once loosely bound host proteins are washed off the resin, PDZ domains bound to PDZ ligand affinity resin should be eluted with concentrated (200-400 μg/ml) PDZ domain peptide ligand. Elution peptides should be natural or synthetic PDZ ligand sequences, six amino acids long, greater than 75% purity and capable of binding to the PDZ domain affinity resin or PDZ domain affinity tag with a dissociation constant (K_D) of 5 μM or less.

Eluted PDZ domain must be concentrated by ultrafiltration in a swinging bucket rotor before dialysis or additional ultrafiltration is performed to exchange buffer and remove bound peptide from the PDZ domain. Failure to remove bound peptide may interfere with the later NHS-coupling reaction, resulting in decreased ligand density of the resin and decreased coupling efficiency.

Due to the time consuming, laborious and potentially hazardous nature of preparing chemically activated chromatography media, commercially available NHS-Activated Agarose should be used for coupling of PDZ domains to resin. To ensure adequate coupling efficiency, coupling must occur in amine free buffer. Following coupling, remaining NHS esters must be quenched with 1 M ethanolamine before the PDZ domain affinity resin is washed and stored. During the coupling procedure, small aliquots of supernatant fractions must be collected for determination of ligand density and coupling efficiency using SDS-PAGE with Coomassie blue staining. Alternatively, the Pierce 660 nm protein assay may be used to measure the concentration of PDZ domain before and after coupling and washing.

Preparation of PDZ domain affinity resin using HaloTag technology—cDNAs encoding PDZ domains to be used for preparation of HaloTag-PDZ domain fusion proteins should be designed as described above for "*Preparation of PDZ domain affinity resin*". Following cloning into the pFN18A vector, in-frame with an N-terminal HaloTag protein, cDNA should be transformed into *E. coli* and protein expressed using auto-induction media.

Cells expressing HaloTag-PDZ domain fusion proteins should be lysed by BugBuster detergent lysis or microfluidization, in buffer containing 10 mM $MgCl_2$ and 2 mM ATP to remove chaperones bound to the HaloTag. Following clarification by centrifugation, lysate containing HaloTag-PDZ domain fusion proteins must be incubated with HaloLink resin for a minimum of 1.5 hours. The HaloTag forms a covalent linkage with the HaloLink resin; therefore, binding times may be extended well beyond 1.5 hours to increase coupling efficiency, as long as there is no noticeable degradation of the HaloTag-PDZ domain protein. After loosely bound host proteins are washed off the resin, the PDZ domain-HaloTag-HaloLink resin should be washed with storage buffer and stored at 4 °C. Storage

should be limited to a maximum of one month, as the covalent linkage between the PDZ domain-HaloTag fusion protein and HaloLink resin is unstable under our storage conditions, resulting in a relatively rapid decline in ligand density (approximately 75% loss of ligand density in 30 days at 4 °C).

Ligand density and coupling efficiency of PDZ domain-HaloTag-HaloLink resin should be determined by treating a small aliquot of resin with TEV protease and quantifying the released PDZ domain using SDS-PAGE with Coomassie blue staining. Alternatively, the concentration of PDZ domain-HaloTag protein in supernatant fractions from the linkage reaction can be assayed by SDS-PAGE with Coomassie blue staining; however, this method has produced highly variable results in our hands. For the TEV proteolysis, it is preferable to use too much TEV protease rather than too little. More efficient cleavage by TEV will produce a more accurate representation of resin ligand density; even under ideal conditions, our cleavage efficiency of immobilized HaloTag fusion proteins rarely exceeds 85%.

Design and Expression of POIs with endogenous PDZ domains or ligands— Expression conditions and construct boundaries for POIs with naturally occurring PDZ domains or ligands should be established by the investigator. To solubilize difficult to express proteins containing PDZ domains or ligands, one should employ a series of techniques as described in (Jana and Deb, 2005; Klock et al., 2008; Makrides, 1996; Sorensen and Mortensen, 2005). To date, PDZ affinity chromatography has been confined to proteins expressed in *E. coli* because of the absence of host cell proteins capable of interacting with PDZ affinity resins (Walkup IV and Kennedy, 2014).

Design and Expression of POIs with PDZ affinity tags—Expression conditions and construct boundaries for POIs fused to PDZ affinity tags should be established by the investigator, however auto-induction media and expression at 37 °C should be attempted first, unless literature precedent suggests otherwise.

PDZ ligand affinity tags can only bind to PDZ domains when placed on the C-terminus of a POI. PDZ ligand affinity tags should contain a short 3 to 5 amino acid linker sequence (e.g. AAA) immediately after the POI, followed by a TEV protease (ENLYFQG) or PreScission protease (LEVLFQGP) cleavage site, and the C-terminal PDZ ligand affinity tag (e.g. SSIESDV).

PDZ domain affinity tags can bind to other PDZ domains or PDZ ligands when placed at the N or C-terminus of a POI, and when placed internally between two proteins (e.g. a POI and a solubility enhancing tag). N-terminal PDZ domain affinity tags should include a TEV protease (or PreScission protease cleavage sequence and a three to five amino acid linker sequence, followed by the POI. C-terminal PDZ domain affinity tags should contain the POI followed by the linker and protease sequences, and finally the C-terminal PDZ domain affinity tag. The placement of internal PDZ domain affinity tags will depend upon the goals of the investigator.

Three tagged variants of each POI should be constructed for PDZ affinity chromatography: 1) N-terminal PDZ domain affinity tag (e.g. PDZ2 domain of PSD-95), 2) C-terminal PDZ

domain affinity tag (e.g. PDZ2 domain of PSD-95), and 3) C-terminal PDZ ligand affinity tag (SSIESDV). These three fusion proteins will allow purification of the POI on both PDZ domain (e.g. PDZ2 domain of PSD-95) and PDZ ligand affinity resin (e.g. GAGSSIESDV) and will increase the chances of identifying a successful affinity tag / affinity resin pair for each unique POI.

Purification of POIs using PDZ affinity chromatography—Design and expression of cDNAs encoding POIs with PDZ affinity tags or endogenous PDZ domains or ligands is discussed above. Following cloning into a suitable vector, cDNA should be transformed into *E. coli* and protein expressed using auto-induction media, unless literature precedent suggests otherwise.

The choice of buffer conditions and lysis method for the preparation of lysate containing POI or partial purification of POI is dependent upon the POI. We have successfully purified POIs with PDZ affinity chromatography using several different buffers (unpublished observation), but have had the most success with HEPES.

Following lysis and clarification by centrifugation, we incubate POIs with PDZ affinity resin for 1 hour at 4 °C with continuous agitation. We have observed maximal binding of POIs to PDZ affinity resin after incubation for 60 to 90 min; extension of incubation times to 2-4 hours resulted in decreased yields of purified POIs. After loosely bound host proteins are washed off the resin, POIs bound to PDZ affinity resin should be eluted with concentrated (200-400 μg/ml) PDZ domain peptide ligand. Elution peptides should be designed as described above. The concentration of the eluted POI can be increased by decreasing the volume of elution buffer, increasing the concentration of peptide from 400 to 800 μg/ml, and/or by incubating the column resin with Peptide Elution Buffer without flow for 20 min before collecting the eluate.

Eluted POIs should be concentrated by ultrafiltration in a swinging bucket rotor before dialysis or additional ultrafiltration is performed to exchange buffer and remove free or bound elution peptides. When purifying POIs fused to a PDZ domain or ligand affinity tag, we have opted to leave the affinity tag attached to the POI, as we have not detected any deleterious effects on POI activity. If removal of affinity tags is necessary, we suggest TEV or PreScission protease. Following concentration and buffer exchange, we quick-freeze aliquots of purified POIs in liquid nitrogen and store at -80 °C.

Troubleshooting

See Table 3.

Anticipated Results

Ligand densities of PDZ ligand affinity resin vary from 20 to 26 nmol peptide/μl resin (20–26 mM), which represents coupling efficiencies of 74–96%, respectively. PDZ ligand affinity resin is stable for approximately 1 year if stored at 4°C.

Ligand densities of PDZ domain affinity resin, prepared by NHS coupling of purified PDZ domains to activated Agarose, vary from 600 to 1300 pmol PDZ domain /μl resin (600-1300

μM), which represents coupling efficiencies of 50–90%, respectively. PDZ domain affinity resin is stable for approximately 6 months if stored at 4°C.

Ligand densities of PDZ domain affinity resin prepared using HaloTag technology vary from 100 -200 pmol PDZ domain /μl of resin (100-200 μM). PDZ domain-HaloTag-HaloLink affinity resin should be stored for a maximum of one month before discarding, as the covalent linkage between the PDZ domain-HaloTag fusion protein and HaloLink resin is unstable under our storage conditions, resulting in a decline in ligand density (approximately 75% loss of ligand density in 30 days at 4 °C).

When purifying PDZ affinity tagged POIs with PDZ affinity resin, we have achieved 500- to 1500-fold purifications with overall yields ranging from 4-81% (36% mean yield), depending on the expression level of the POI and the affinity of its PDZ tag for PDZ affinity resin. The concentration of affinity purified POIs has ranged from 5.8-82 μM (27 μM mean concentration) prior to concentration by ultrafiltration. All PDZ domain or ligand affinity tagged POIs that we have purified with PDZ affinity resin have been greater than 95% pure when assayed by SDS-PAGE and Coomassie blue staining. Enzymes purified using PDZ affinity chromatography have also been recovered in folded and functional form, even when fused to PDZ affinity tags.

When purifying POIs that have endogenous PDZ domains or ligands with PDZ affinity resin, we have achieved 500- to 1500-fold purifications with overall yields ranging from 4-96% (30% mean yield), depending on the expression level of the POI and its affinity for PDZ affinity resin. The concentration of affinity purified POIs has ranged from 0.2-9 μM (4 μM mean concentration) prior to concentration by ultrafiltration. All PDZ domain or ligand affinity tagged POIs that we have purified with PDZ affinity resin have been greater than 70% pure when assayed by SDS-PAGE and Coomassie blue staining, with the majority being greater than 95% pure.

The stability of POIs purified by PDZ affinity chromatography is variable; however, the proteins we have purified have remained stable for over six months if flash frozen in liquid nitrogen and stored at −80°C in an appropriate buffer. Purified POIs must be aliquoted and quick-frozen in liquid nitrogen for storage and should be quick-thawed in cold water and placed on ice before use.

Time Considerations

Basic Protocol 1 requires PDZ ligand design (0-6 hours) and synthesis (0.5-3 weeks) and NHS coupling to chemically activated Agarose (3 hours) to prepare PDZ ligand affinity resin. To determine the ligand density of the resin, supernatant fractions may be dialyzed (12-24 hours) and quantified by BCA or A_{280} assay (1 hour) or quantified via the Pierce 660 nm assay (1 hour; no dialysis required) if the ligand sequence contains one or more lysine residues.

Basic Protocol 2 requires design of cDNAs encoding PDZ domain (0-6 hours), followed by synthesis (2 weeks) or cloning (1-2 weeks). Transformation of *E. coli* cells with cDNA and expression of PDZ domain (3 days) followed by purification on PDZ ligand affinity resin

(12 hours; 24 hours if using dialysis) and NHS coupling to chemically activated Agarose (12-18 hours) are required prepare PDZ domain affinity resin. To determine the ligand density of the resin, supernatant fractions may be dialyzed (12-24 hours) and quantified by BCA or A_{280} assay (1 hour) or quantified via SDS-PAGE and Coomassie staining (2 hours to run gels; 2-14 hours for staining and imaging; no dialysis required).

Alternate Protocol 2 requires design of cDNAs encoding PDZ domain (0-6 hours), followed by synthesis (2 weeks) and/or cloning (1-2 weeks) into pFN18A. The next step requires transformation of *E. coli* cells with cDNA and expression of HaloTag-PDZ domain fusion proteins (2 days) followed by purification/coupling to HaloLink resin (6.5 hours). To determine the ligand density of the resin, TEV proteolysis of a resin aliquot (2.5 hours) is followed by SDS-PAGE and Coomassie staining (2 hours to run gels; 2-14 hours for staining and imaging).

Basic Protocol 3 requires cloning and expression of the POI containing PDZ domains or PDZ domain-binding ligands (variable) and purification on PDZ affinity resin (5 hours; 24 hours if using dialysis) and regeneration of PDZ affinity resin (0.5 hour). Analysis of the purification fractions by SDS-PAGE and Coomassie staining (2 hours to run gels; 2-14 hours for staining and imaging), Western Blotting (12 - 24 hours), or activity assays (variable) can be carried out at the investigator's discretion.

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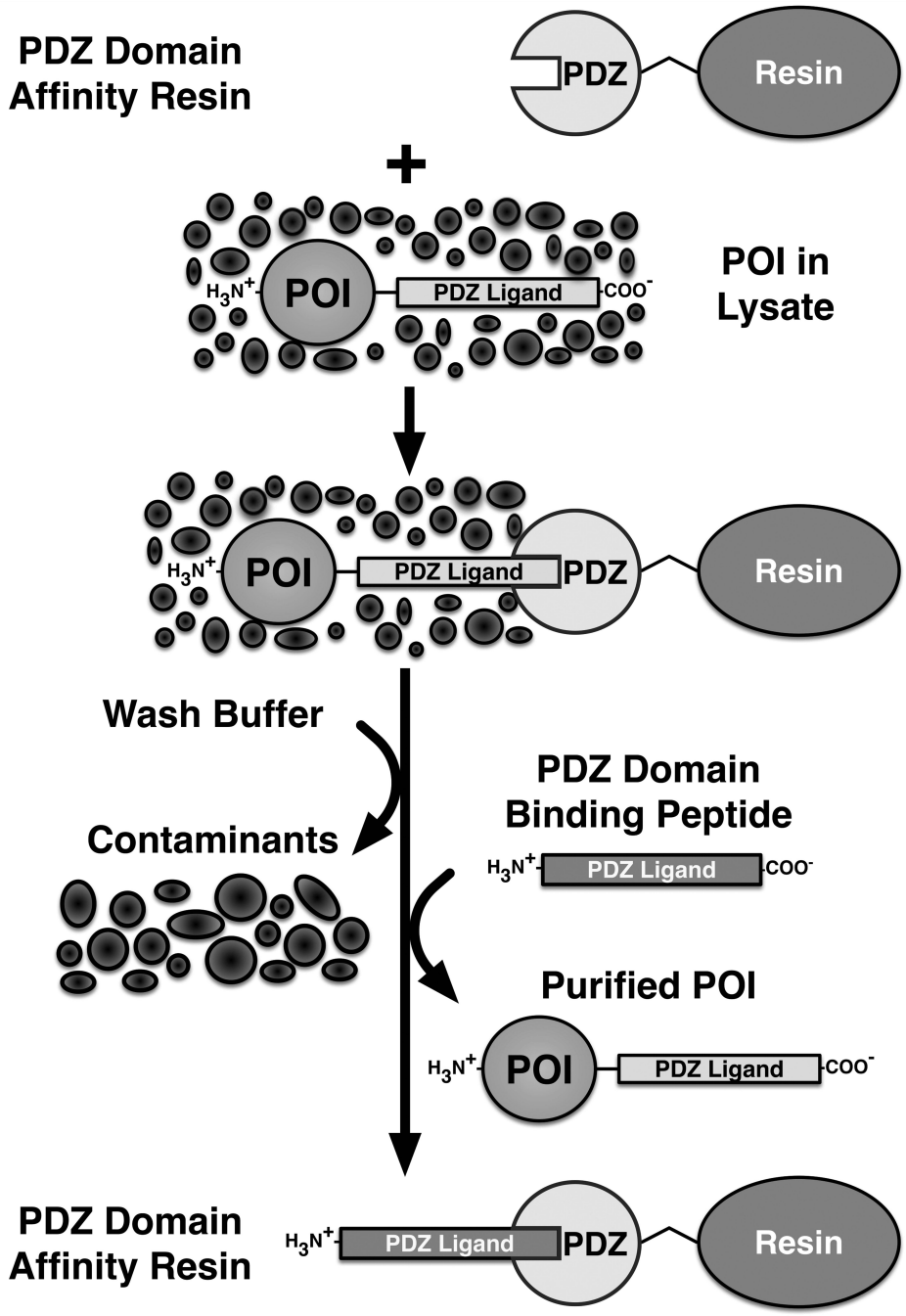

Figure 1. PDZ Domain Affinity Chromatography Purification Scheme I: Purification of POI Containing a PDZ Domain Peptide Ligand Tag Using PDZ Domain Affinity Resin

A POI containing a naturally occurring or recombinantly appended PDZ domain peptide ligand, can be captured from a cellular lysate by binding to a PDZ domain coupled to a solid support resin. Extensive washing of the captured POI removes cellular contaminants. Free PDZ domain peptide ligand is then added to competitively elute the captured POI. Figure and legend are reproduced (with alterations) from (Walkup IV and Kennedy, 2014).

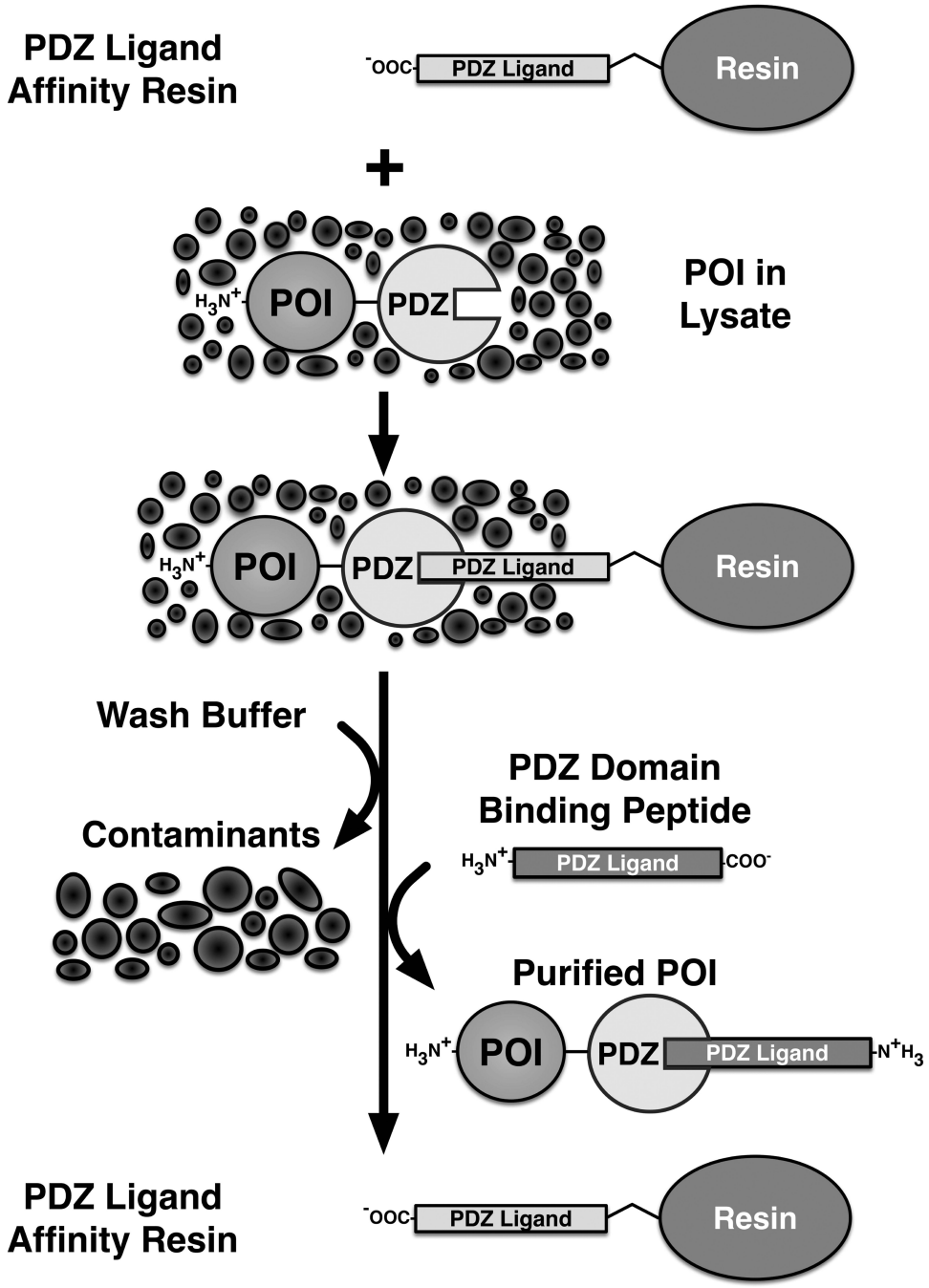

Figure 2. PDZ Domain Affinity Chromatography Purification Scheme II: Purification of POI Containing a PDZ Domain Tag Using PDZ Domain Peptide Ligand Affinity Resin

A POI containing a naturally occurring or recombinantly appended PDZ domain can be captured from a cellular lysate by binding to a PDZ domain peptide ligand coupled to a solid support resin. Extensive washing of the captured POI removes cellular contaminants. As in Figure 1, free PDZ domain peptide ligand is then added to competitively elute the captured POI. Figure and legend are reproduced (with alterations) from (Walkup IV and Kennedy, 2014).

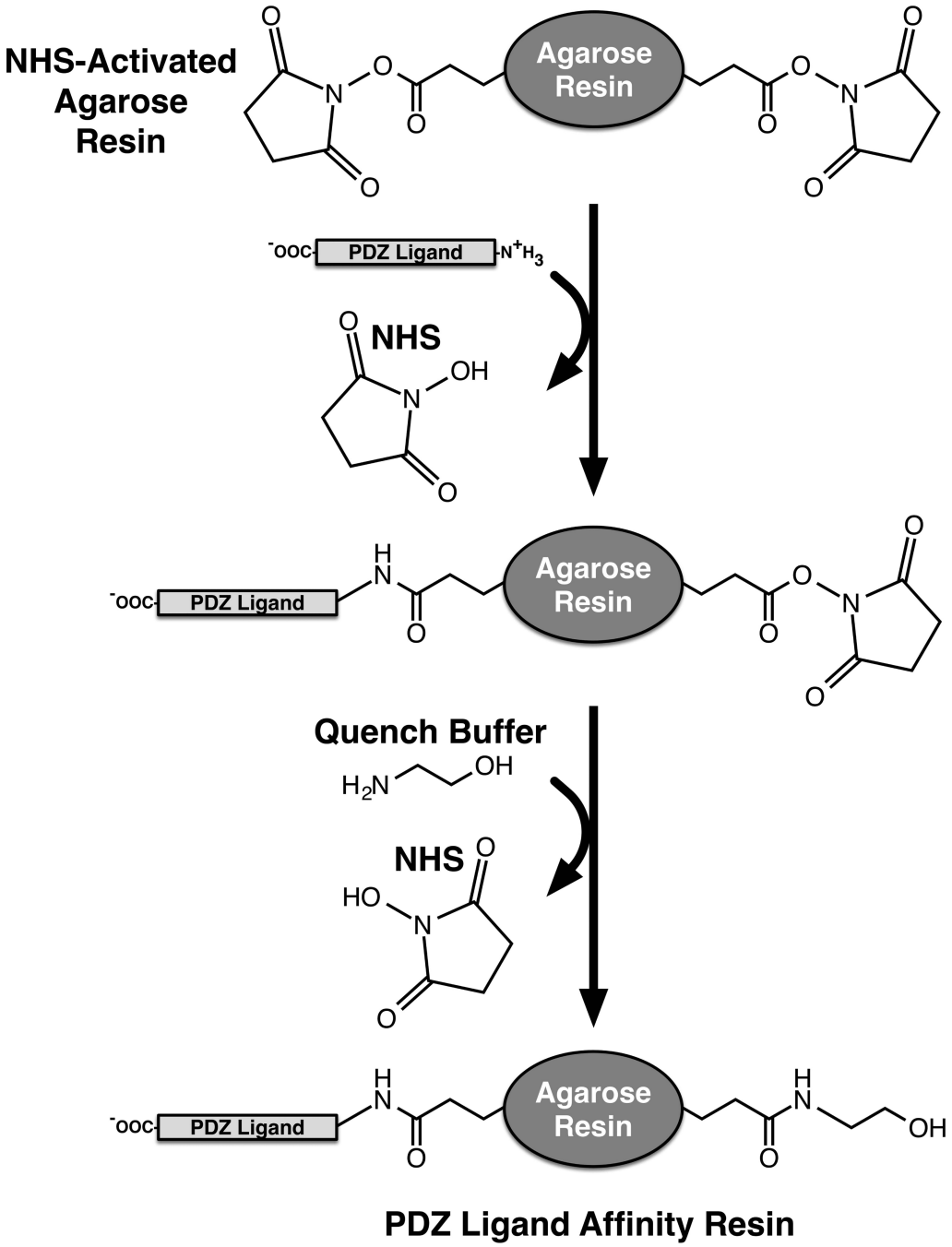

Figure 3. Preparation of PDZ Ligand Affinity Resin

A peptide ligand for a PDZ domain is incubated with NHS-Activated Agarose resin to form a covalent amide linkage between the Agarose resin and the N-terminus of the peptide ligand. Following washing to remove released NHS, the remaining NHS reactive groups on the resin are blocked with Quenching Buffer containing ethanolamine. After additional washing to remove released NHS and ethanolamine, PDZ ligand affinity resin is ready for use in PDZ affinity chromatography experiments.

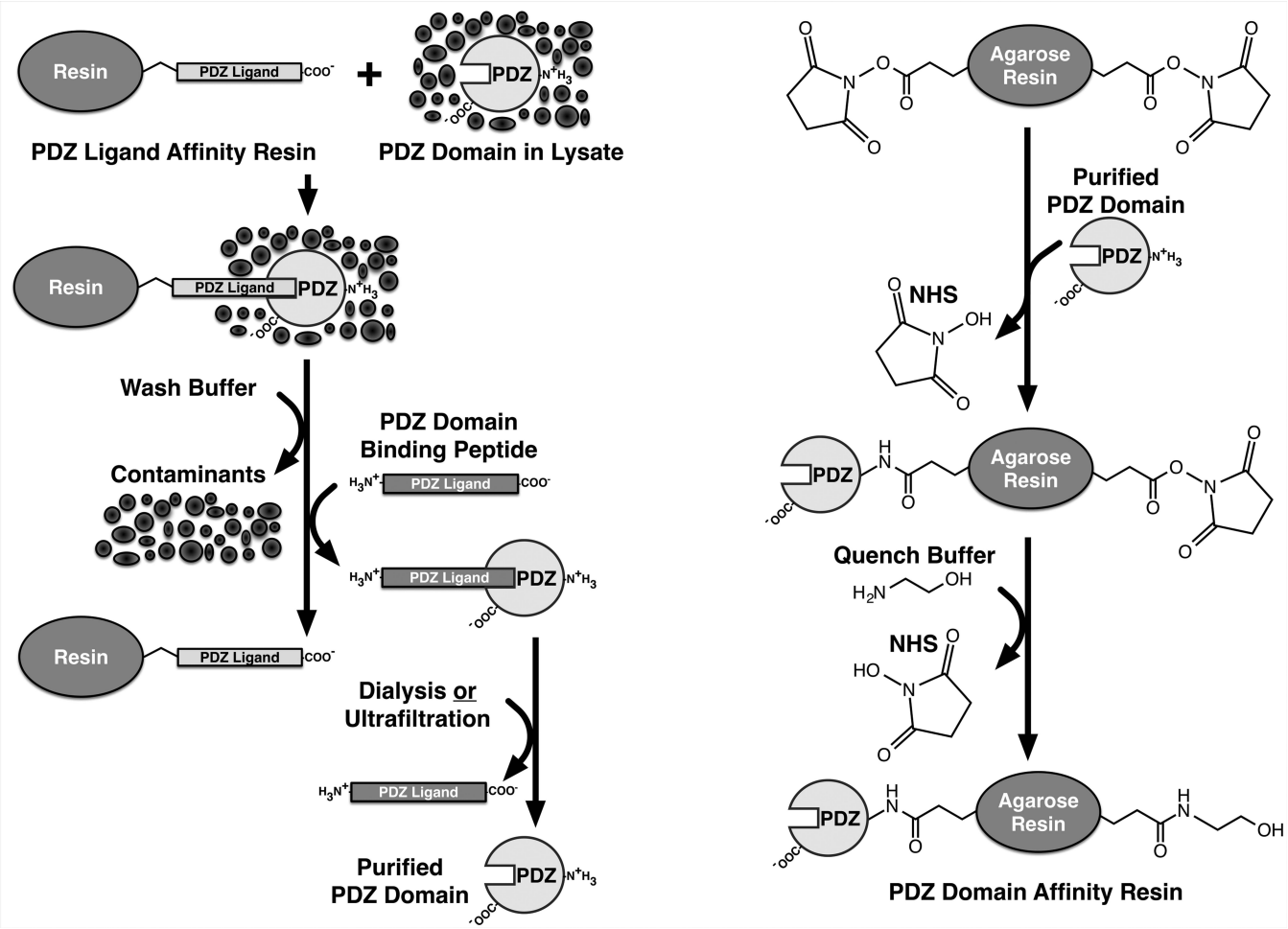

Figure 4. Preparation of PDZ Domain Affinity Resin

(Left) An expressed PDZ domain is captured from a cellular lysate by binding to PDZ domain ligand affinity resin. Extensive washing of the captured PDZ domain and resin removes cellular contaminants. Free PDZ domain peptide ligand is then added to competitively elute the captured PDZ domain. Bound peptide ligand is removed from the PDZ domain by dialysis or ultrafiltration.

(Right) The purified PDZ domain is then incubated with NHS-Activated Agarose resin to form a covalent amide linkage between the Agarose resin and primary amines present on lysine residues or the N-terminus of the PDZ domain. Following washing to remove released NHS, the remaining NHS reactive groups on the resin are blocked with Quenching Buffer containing ethanolamine. After additional washing to remove released NHS and ethanolamine, PDZ domain affinity resin is ready for use in PDZ affinity chromatography experiments.

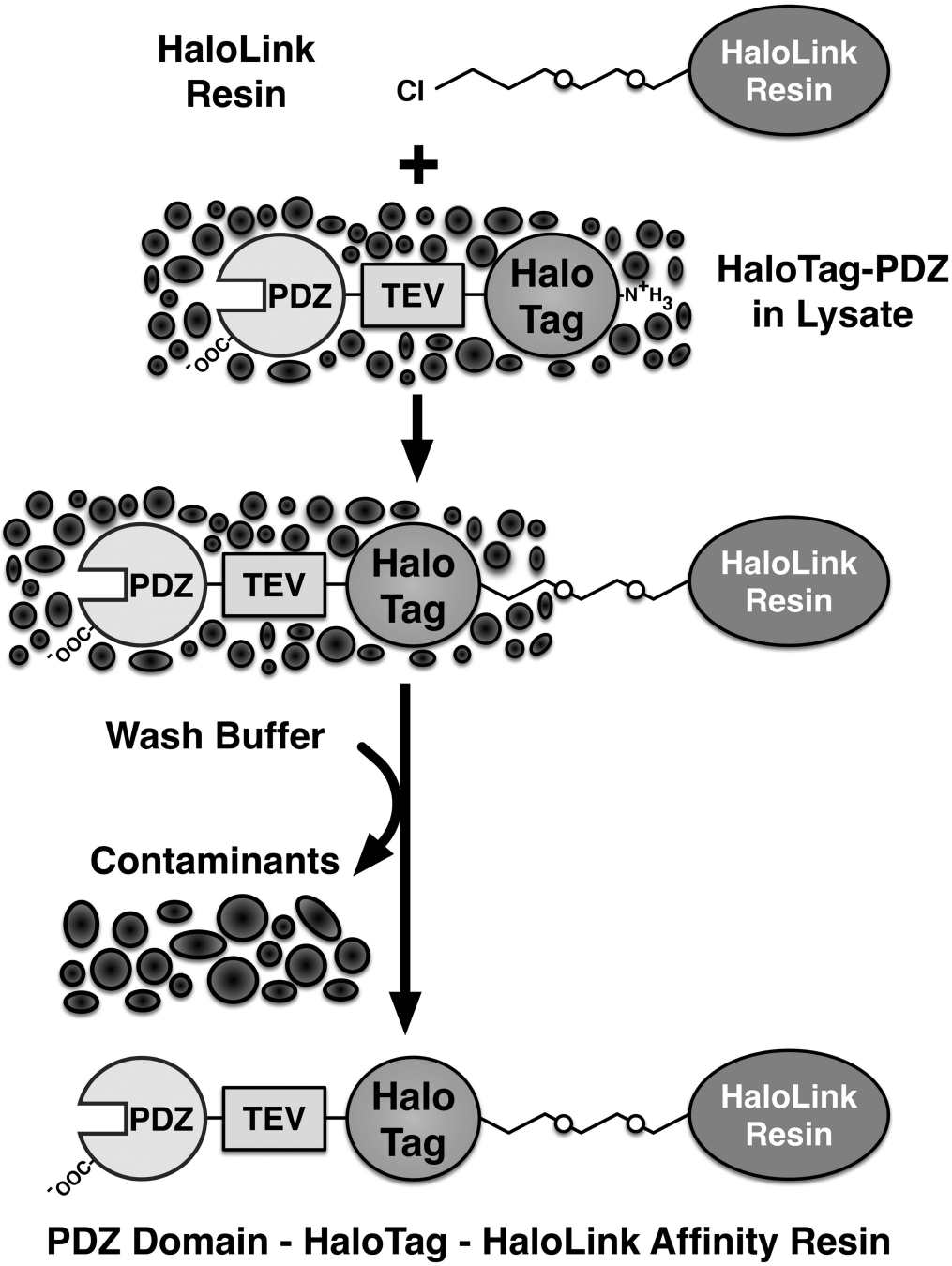

Figure 5. Preparation of PDZ Domain-HaloTag-HaloLink Affinity Resin

An expressed fusion protein containing an N-terminal HaloTag and PDZ domain can be covalently captured from a cellular lysate by binding to HaloLink resin. Following extensive washing of the captured fusion protein to remove cellular contaminants, the PDZ domain-HaloTag-HaloLink affinity resin is ready for use in PDZ affinity chromatography experiments.

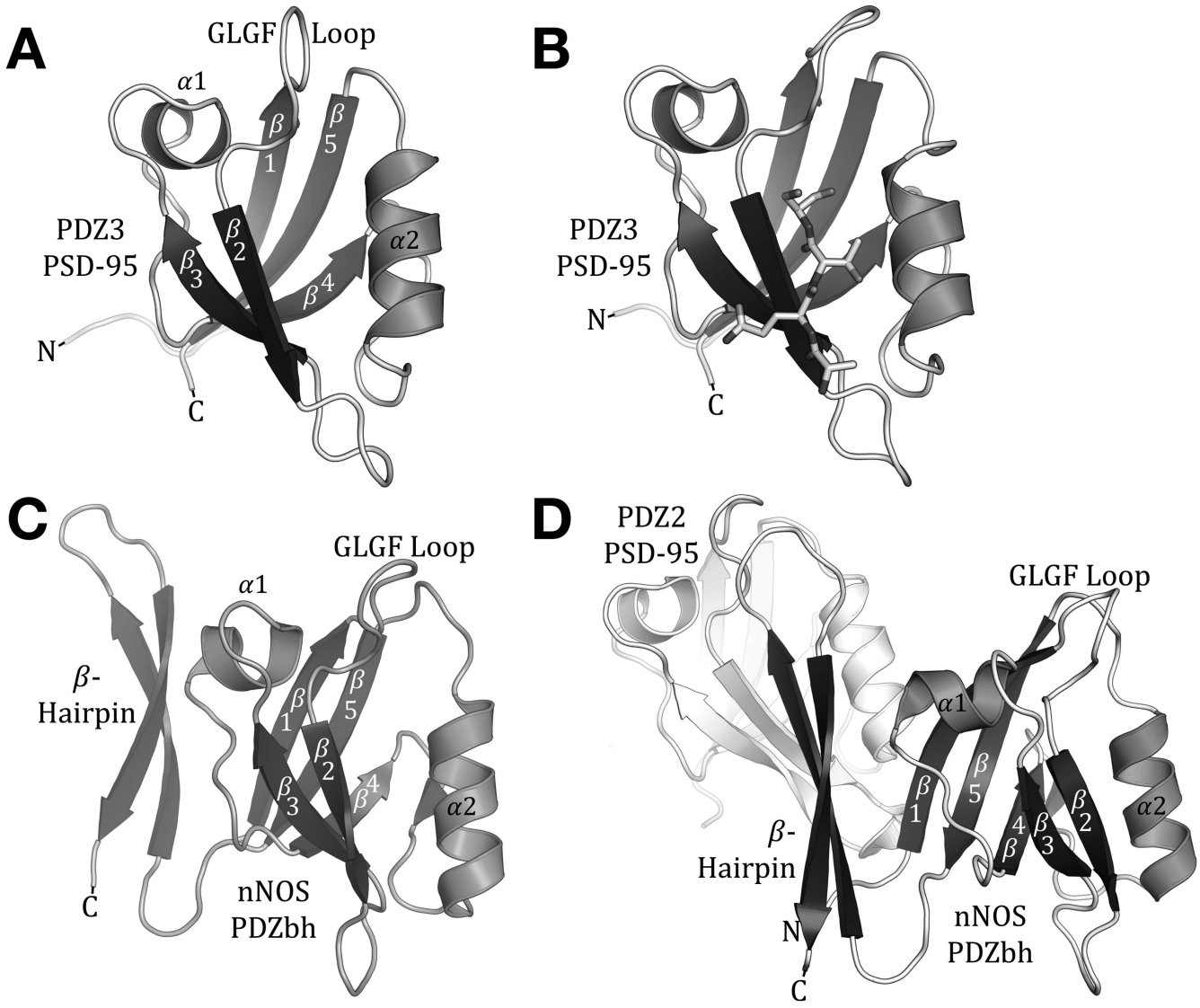

Figure 6. Structures of PDZ Domains in Isolation and Complexed With Ligands

(**A**),(**B**) Ribbon diagram of the three dimensional structure of the PDZ3 domain from PSD-95 (RCSB ID: 1BFE) in isolation (**A**) and bound to the C-terminal peptide ligand (KQTSV) from CRIPT (RCSB ID: 1BE9) (**B**). PDZ domains consist of five β-strands (β1-β5) arranged in an antiparallel β-barrel flanked by two α-helices (α1-α2). The peptide ligand (Drawn in stick form) inserts in a hydrophobic pocket lined by the β2 strand, β1-β2 loop and the α2 helix and forms an antiparallel β-sheet with the β2 strand. The β1-β2 loop is required for recognition of the C-terminal carboxylate group of the ligand, and is referred to as the carboxylate-binding loop or GLGF loop. (**C**),(**D**) Ribbon diagram of the three dimensional structure of the PDZ-β-Hairpin (PDZbh) domain from nNOS (RCSB ID: 1QAU) in isolation (**C**) and in complex with the PDZ domain from syntrophin (RCSB ID: 1QAV) (**D**). The PDZbh has a polarized structure with two distinct faces: a ligand binding pocket and β-

hairpin face. This allows the ligand binding pocket of the nNOS PDZbh to bind a ligand while its β-hairpin extension is complexed with the syntrophin PDZ domain (White ribbon).

Table 1

PDZ domain and C-terminal ligand pairs used successfully for PDZ affinity chromatography

PDZ domains and PDZ ligand sequences listed in the same row comprise PDZ ligand-PDZ domain pairs, and can be used interchangeably as PDZ affinity tags or on PDZ affinity resin. Ligand sequences listed in the right hand column can be used to dissociate PDZ ligand-PDZ domain complexes listed in the same row (Kimple and Sondek, 2002; Walkup IV and Kennedy, 2014).

PDZ Domain Sequence(s)	**PDZ Ligand Sequence(s)**	**PDZ Ligand Sequence(s) Used for Elution from PDZ Affinity Resin**
>*Mus musculus* PSD-95 PDZ1 (61-151) Q62108 MEYEEITLERGNSGLGFSIA GGTDNPHIGDDPSIFITKIIP GGAAAQDGRLRVNDSILFV NEVDVREVTHSAAVEALKE AGSIVRLYVMRR	>PDZ ligand affinity tag SSIESDV	>GluN2B C-terminus SIESDV >Synthetic Peptides* SIETEV KIETEV
>*Mus musculus* PSD-95 PDZ2 (155-249) Q62108; PDZ domain affinity tag AEKIIEIKLIKGPKGLGFSIAG GVGNQHIPGDNSIYVTKIIEG GAAHKDGRLQIGDKILAVNS VGLEDVMHEDAVAALKNTY DVVYLKVAKPSNA	>GluN2B C-terminus EKLSSIESDV >GluN2B C-terminus KLSSIESDV >GluN2B C-terminus LSSIESDV >GluN2B C-terminus; PDZ ligand affinity tag SSIESDV >GluN2B C-terminus SIESDV >GluN2B C-terminus IESDV	>GluN2B C-terminus SIESDV >Synthetic Peptides* SIETEV KIETEV
>*Mus musculus* PSD-95 PDZ1-2 (61-249) Q62108 MEYEEITLERGNSGLGFSIA GGTDNPHIGDDPSIFITKIIP GGAAAQDGRLRVNDSILFV NEVDVREVTHSAAVEALKE AGSIVRLYVMRRKPPAEKIIE IKLIKGPKGLGFSIAGGVGN QHIPGDNSIYVTKIIEGGAAH KDGRLQIGDKILAVNSVGLE DVMHEDAVAALKNTYDVVY LKVAKPSNA	>GluN2B C-terminus EKLSSIESDV >GluN2B C-terminus KLSSIESDV >GluN2B C-terminus LSSIESDV >GluN2B C-terminus; PDZ ligand affinity tag SSIESDV >GluN2B C-terminus SIESDV >GluN2B C-terminus IESDV	>GluN2B C-terminus (PDZ1 or PDZ2) SIESDV >Synthetic Peptides (PDZ1 or PDZ2)* SIETEV KIETEV
>*Drosophila melanogaster* InaD PDZ (11-107) CG3504** AGELIHMVTLDKTGKKSFGI CIVRGEVKDSPNTKTTGIFIK GIVPDSPAHLCGRLKVGDRI LSLNGKDVRNSTEQAVIDLI KEADFKIELEIQTFDK	>*Drosophila melanogaster* NorpA (1091-1095) CG3620** TEFCA	>Reducing Agent** 20-100 mM DTT

* Synthetic peptide sequences were taken from (Lim et al., 2002)

** Data from (Kimple and Sondek, 2002).

Table 2
Neuronal proteins purified using PDZ Affinity Chromatography

Heterologously expressed neuronal proteins were purified using PDZ affinity resin and ligand sequences listed in the same row (Walkup IV and Kennedy, 2014).

Purified Neuronal Protein	PDZ Affinity Resin	PDZ Ligand Sequence(s) Used for Elution from PDZ Affinity Resin
>6x Histidine tagged *Rattus norvegicus* synGAP (103-1293) Q9QUH6	>*Mus musculus* PSD-95 PDZ1 (61-151) Q62108 >*Mus musculus* PSD-95 PDZ2 (155-249) Q62108 >*Mus musculus* PSD-95 PDZ3 (302-402) Q62108	>GluN2B C-terminus (PDZ1 or PDZ2) SIESDV >Synthetic Peptides (PDZ1 or PDZ2)* SIETEV KIETEV >CRIPT C-terminus (PDZ3) YKQTSV >Synthetic peptide (PDZ3)** KKETAV
>Maltose Binding Protein - *mus musculus* GluN2B Tail (842-1482) Q01097 >*Mus musculus* nNOS PDZ (11-129) Q9Z0J4	>*Mus musculus* PSD-95 PDZ2 (155-249) Q62108	>GluN2B C-terminus SIESDV >Synthetic Peptides* SIETEV KIETEV
>*Rattus norvegicus* CRIPT Q792Q4	>*Mus musculus* PSD-95 PDZ3 (302-402) Q62108	>CRIPT C-terminus YKQTSV >Synthetic peptide** KKETAV
>*Homo sapiens* cypin Q9Y2T3 >Maltose Binding Protein - *Homo sapiens* cypin Q9Y2T3	>*Mus musculus* PSD-95 PDZ1-2 (61-249) Q62108	>GluN2B C-terminus (PDZ1 or PDZ2) SIESDV >Synthetic Peptide (PDZ1 or PDZ2)* SIETEV KIETEV

* Synthetic peptide sequences were taken from (Lim et al., 2002)

** Synthetic peptide sequence was taken from (Saro et al., 2007)

Table 3

Common Problems and Solutions

Problem	Cause(s)	Solution(s)
Basic Protocol 1: Preparation of PDZ ligand affinity resin		
Low coupling efficiency	- Primary amine buffer not removed before coupling	- Dialyze or desalt ligand sample to completely remove primary amine buffer or use alternate buffer.
	- Buffer composition is not optimal	- Check buffer composition and measure pH at correct temperature.
	- NHS-activated resin is old/ degraded	- Purchase or prepare new NHS-activated resin.
	- Amine groups on ligand are occluded	-Use alternate ligand sequence. -Use alternate coupling buffer.
Ligand not soluble in coupling buffer	- Ligand is hydrophobic	- Dissolve ligand in coupling buffer containing up to 4 M guanidinium, 20% DMSO or organic solvents.
	- Buffer composition is not optimal	-Check buffer composition and measure pH at correct temperature. -Adjust pH of buffer; coupling should be performed between pH 7 and 9 if possible.
Basic Protocol 2: Preparation of PDZ domain affinity resin		
Low coupling efficiency	- Primary amine buffer not removed before coupling	- Dialyze or desalt PDZ domain sample to completely remove primary amine buffer or use alternate buffer.
	- Buffer composition is not optimal	- Check buffer composition and measure pH at correct temperature.
	- NHS activated resin is old/ degraded	- Purchase or prepare new NHS-activated resin.
	- Amine groups on PDZ domain are occluded	-Use alternate PDZ domain sequence. -Use alternate coupling buffer.
PDZ domain not soluble in coupling buffer	- Domain is hydrophobic	- Dissolve domain in coupling buffer containing non-ionic detergent.
	- Buffer composition is not optimal	-Check buffer composition and measure pH at correct temperature. -Adjust pH of buffer; coupling should be performed between pH 7 and 9 if possible.
PDZ domain does not bind to ligand affinity resin or elutes in wash buffer	- Problem with vector construction	- Verify vector DNA sequence.
	- Ligands on PDZ affinity resin are occluded or damaged	-Prepare new ligand affinity resin. -Increase ligand spacer length. -Prepare resin using HaloTag technology.
	- PDZ domain is aggregated or misfolded	-Reduce expression temperature. -Express in alternate cell line or promoter. -Systematically truncate POI at N and/or C-termini. -Add detergents for solubilization.
	- Incompatible PDZ affinity tag and resin	- Check that PDZ domain and ligand affinity resin are compatible. If not, select new PDZ domain and/or ligand affinity resin.
	- Buffer composition is not optimal	- Check buffer composition and measure pH at correct temperature.
	- PDZ domain is degraded during lysis or purification	-Perform lysis or initial purification steps more quickly. -Increase amount of protease inhibitor. -Use alternate PDZ domain.
	- Ligand affinity resin is degraded or has not been regenerated	-Prepare new ligand affinity resin. -Regenerate existing ligand affinity resin or wash with high salt (1M NaCl) to remove nonspecifically bound material.

Problem	Cause(s)	Solution(s)
Alternate Protocol 2: Preparation of PDZ domain affinity resin using HaloTag® technology		
HaloTag-PDZ fusion protein is not expressed, or is truncated	- Problem with vector construction	- Verify vector DNA sequence.
	- Suboptimal culture and growth conditions	- Optimize growth conditions including media composition, temperature and induction conditions.
	- HaloTag-PDZ fusion not soluble	- Check total and soluble cellular lysate for POI. If insoluble, alter lysis conditions, growth conditions, construct boundaries, PDZ domain or location.
	- Host cell protein co-migrates with HaloTag-PDZ fusion on SDS-PAGE gel	- Use different percentage acrylamide gel to separate bands or use HaloTag TMR ligand for specific detection of fusion protein.
	- PDZ domain alters fusion protein expression	-Use alternate PDZ domain. -Change PDZ domain location in fusion protein (N or C-terminal).
	- Fusion protein is cleaved by host cell protease	-Perform lysis or initial purification steps more quickly. -Increase amount of protease inhibitor. -Use alternate linker region or PDZ domain. -Change PDZ domain location in fusion protein (N or C-terminal). -Express at lower temperature and/or with molecular chaperones.
HaloTag - PDZ domain does not bind to HaloLink resin	- Problem with vector construction	- Verify vector DNA sequence.
	- HaloTag is occluded by structure of PDZ domain or by interaction with host cell proteins	-Use alternate PDZ domain. -Change PDZ domain location in fusion protein (N or C-terminal). -Systematically truncate PDZ domain at N and/or C-termini. -Add small amount of nonionic detergent to potentially improve tag accessibility. -Add 10 mM $MgCl_2$ and 2 mM ATP to remove bound chaperones. -Increase ionic strength of purification buffer to reduce non-specific interactions with host proteins.
	- Fusion protein is aggregated or misfolded	-Reduce expression temperature. -Express in alternate cell line or promoter. -Systematically truncate PDZ domain at N and/or C-termini. -Add detergents for solubilization.
	- Buffer composition is not optimal	- Check buffer composition and measure pH at correct temperature.
	- Fusion protein is degraded during lysis or purification	-Perform lysis or initial purification steps more quickly. -Increase amount of protease inhibitor. -Use alternate PDZ domain. -Change PDZ domain location in fusion protein (N or C-terminal).
	- HaloLink resin has degraded or not equilibrated with buffer	-Purchase new HaloLink resin. -Equilibrate resin with purification buffer.
	- Fusion protein was damaged during lysis or inactivated by lysis buffer	-For lysis by sonication, conduct a time course assay to determine minimum time required to disrupt cells, that maintains HaloTag activity. -If using non-ionic detergent for lysis, use alternative detergents or use sonication or microfluidization instead.
	- Insufficient binding time	- Increase binding time to overnight.
PDZ domain cannot be cleaved from HaloTag-HaloLink resin using TEV protease	- HaloTag-PDZ fusion protein did not express or was insoluble	- Check total and soluble cellular lysate for fusion protein. If insoluble, alter lysis conditions, growth conditions, construct boundaries, PDZ domain or location.
	- Fusion protein is degraded during lysis or purification	-Perform lysis or initial purification steps more quickly. -Increase amount of protease inhibitor. -Use alternate PDZ domain.

Problem	Cause(s)	Solution(s)
		-Change PDZ domain location in fusion protein (N or C-terminal).
	- TEV cleavage is not efficient	-Verify vector DNA sequence and check TEV cleavage site for correct sequence. -Increase TEV cleavage time to overnight. -Increase amount of TEV in reaction mixture. -Add DTT or TCEP and remove divalent cations and detergents. -TEV protease may have degraded. Purchase new TEV protease.
Basic Protocol 3: Purification of POIs using PDZ affinity chromatography		
POI is not expressed, or is truncated	- Problem with vector construction	- Verify vector DNA sequence.
	- Suboptimal culture and growth conditions	- Optimize growth conditions including media composition, temperature and induction conditions.
	- POI not soluble	- Check total and soluble cellular lysate for POI. If insoluble, alter lysis conditions, growth conditions, construct boundaries or PDZ affinity tag type or location.
	- Host cell protein co-migrates with POI on SDS-PAGE gel	- Use different percentage acrylamide gel to separate bands or use Western Blotting for specific detection of POI.
	- PDZ affinity tag alters POI expression	-Use alternate PDZ affinity tag and/or resin. -Change affinity tag location in fusion protein (N or C-terminal).
	- POI is cleaved by host cell protease	-Perform lysis or initial purification steps more quickly. -Increase amount of protease inhibitor. -Use alternate PDZ affinity tag and/or resin. -Change affinity tag location in fusion protein (N or C-terminal). -Express at lower temperature and/or with molecular chaperones.
POI does not bind to PDZ affinity resin or elutes in wash buffer	- Problem with vector construction	- Verify vector DNA sequence.
	- PDZ affinity tag is occluded by structure of POI or by interaction with host cell proteins	-Use alternate PDZ affinity tag and/or resin. -Change affinity tag location in fusion protein (N or C-terminal). -Add small amount of nonionic detergent to potentially improve tag accessibility. -Add 10 mM $MgCl_2$ and 2 mM ATP to remove bound chaperones. -Increase ionic strength of purification buffer to reduce non-specific interactions with host proteins.
	- Ligands on PDZ affinity resin are occluded or damaged	-Prepare new PDZ affinity resin. -Increase spacer length. -Prepare resin using HaloTag technology.
	- POI is aggregated or misfolded	-Reduce expression temperature. -Express in alternate cell line or promoter. -Systematically truncate POI at N and/or C-termini. -Add detergents for solubilization.
	- Incompatible PDZ affinity tag and resin	- Check that PDZ domain and ligand affinity resin are compatible. If not, select new PDZ domain and/or ligand affinity resin.
	- Buffer composition is not optimal	- Check buffer composition and measure pH at correct temperature.
	- POI or affinity tag is degraded during lysis or purification	-Perform lysis or initial purification steps more quickly. -Increase amount of protease inhibitor. -Use alternate PDZ affinity tag and/or resin. -Change affinity tag location in fusion protein (N or C-terminal).
	- Affinity resin is degraded or has not been regenerated	-Prepare new PDZ affinity resin.

Problem	Cause(s)	Solution(s)
		-Regenerate existing PDZ affinity resin or wash with high salt (1 M NaCl) to remove nonspecifically bound material.
	- Insufficient binding time	- Increase binding time from 1 hour to overnight.
POI eluted from PDZ affinity resin is pure but dilute	- Elution volume too large	- Reduce elution volume.
	- Elution was performed too rapidly	- Add peptide elution buffer to affinity resin and cap column for 15-20 min before draining.
	- POI still bound to column	-Increase concentration of free ligand in peptide elution buffer; add a second ligand or use alternate ligand. -Decrease elution buffer pH or increase ionic strength. -If POI can be refolded, elute with denaturant and refold POI following elution.
	- Low POI expression level	- Increase the amount of lysate containing POI incubated with fixed amount of PDZ affinity resin.
	- Low binding capacity of affinity resin for POI	- Increase the amount of PDZ affinity resin incubated with a fixed amount of lysate containing POI.
	- Affinity resin is degraded or has not been regenerated	-Prepare new PDZ affinity resin. -Regenerate existing PDZ affinity resin or wash with high salt (1 M NaCl) to remove nonspecifically bound material.
POI eluted from PDZ affinity resin is impure	- Insufficient washing	- Use larger volume of wash buffer.
	- Buffer composition is not optimal	- Check buffer composition and measure pH at correct temperature.
	- Host cell proteins bind to PDZ affinity resin	-Increase ionic strength of buffer to reduce non-specific ionic interactions. -Add small amount of non-ionic detergent. -Add glycerol to reduce non-specific hydrophobic interactions. -Express POI in different cell line. -Use alternate PDZ affinity tag and/or resin.
	- Low affinity of POI for affinity resin	-Use alternate PDZ affinity tag and/or resin. -Change affinity tag location in fusion protein (N or C-terminal). -Check the composition of all buffers and measure pH at correct temperature.
	- POI is interacting with host cell proteins	-Increase ionic strength of purification buffer to reduce non-specific ionic interactions with host proteins. -Add small amounts of non-ionic detergent. -Add glycerol to reduce non-specific hydrophobic interactions. -Express POI in alternate cell line. -Add 10 mM $MgCl_2$ and 2 mM ATP to remove bound chaperones.
	- Resin was used in large excess	- Estimate the concentration of POI in lysate and increase amount of lysate incubated with fixed amount of PDZ affinity resin.
	- Disulfide bond formation between impurities and POI	- Increase concentration of DTT, TCEP or β-mercaptoethanol in wash and elution buffer.
POI is inactive	- POI was damaged during lysis or inactivated by lysis buffer	-For lysis by sonication, conduct a time course assay to determine minimum time required to disrupt cells, that maintains POI activity. -If using non-ionic detergent for lysis, use alternative detergents or use sonication or microfluidization instead.
	- PDZ affinity tag inhibits POI activity	-Use alternate PDZ affinity tag and/or resin. -Change affinity tag location in fusion protein (N or C-terminal). -Remove tag by proteolysis following purification.
	- POI misfolded	-Elute protein from affinity resin with denaturant and refold.

Problem	Cause(s)	Solution(s)
		-Reduce expression temperature. -Express in alternate cell line or promoter. -Systematically truncate POI at N and/or C-termini.
	- POI requires post-translational modification for activity	-Express POI in alternate cell line. -Coexpress POI with required enzymes for post-translational modification.